THE SUBCELLULAR LOCALIZATION OF THE UNCHARACTERIZED HUMAN DISEASE-ASSOCIATED PROTEIN POTASSIUM CHANNEL TETRAMERIZATION DOMAIN 13 (KCTD13)

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

April 2016

ABSTRACT

Human KCTD (potassium (K+) channel tetramerization domain (KCTD) proteins make up a family of 26 largely uncharacterized human proteins containing a single N-terminal BTB/POZ domain most closely related to the T1 domain of potassium channels. KCTDs have been implicated in a variety of human diseases including cancer and neurological disorders. KCTD13 (also known as BACURD1, PDIP1 and POLDIP1), encoded on human chromosome 16p11.2, has been recently implicated in human disease, including neurological disorders and autism spectrum disorder (ASD). However, the molecular mechanisms are unknown. To gain insight on the function of KCTD13, and the family of uncharacterized KCTD proteins, the subcellular localization of expressed KCTD13 was determined, leading to the hypothesis that KCTD13 may have a role in the a protein quality control pathway possibly required for mitochondrial maintenance. Localization of epitope-tagged KCTD13 in mammalian cells was analyzed by immunofluorescence microscopy. Results indicate that KCTD13 forms distinct cytoplasmic structures that co-localize with the E3-ubiquitin ligase Cullin-3 (Cul-3) and mitochondrial organelles. Interestingly, uncoupling of mitochondria by treatment of cells with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) intensifies the co-localization of expressed KCTD13 with the mitochondria and cul-3, suggesting a potential role in the clearance of damaged mitochondrial organelles or components thereof. Further supporting this hypothesis, KCTD13 also co-localizes with autophagy markers LC3, ATG12 and ATG13. KCTD13 also partially localizes with the endosome markers RAB5 and prominently localizes with late endosome marker RAB7. Taken together, these findings are consistent with a potential role of KCTD13 in mitochondrial homeostasis

and provide a new perspective in the understanding of molecular mechanisms contributing to ASD and other diseases in which KCTD13 mutations may contribute.

Primary Reader: J. Marie Hardwick, PhD, Thesis Advisor

Secondary Reader: Isabelle Coppens, PhD

ACKNOWLEDGEMENTS

Two years went by so quickly, going so quickly I barely knew it was almost over. During this fast-paced two-year Master's journey I have learned a great deal about science, infectious diseases and laboratory techniques, and I learned even more about myself. I have struggled with the frustrations of failed experiments that caused me to question my abilities inside and outside the laboratory. Despite early self-doubts, I pushed through to make new discoveries that contribute valuable new information to science, and I sincerely appreciate the efforts of the many people who stood behind me and supported me throughout this journey to a new level of self-confidence.

I would like to express my deep gratitude to my adviser Dr. J. Marie Hardwick. She has believed in me from the very beginning, taking me as one of her students despite my lack of wet laboratory experience. She has never given up on me and for that I am very thankful. I probably would have not made it this far if not for her encouragement, patience and understanding. Along with Dr. Hardwick, the members of the Hardwick Laboratory have also been a great source of support.

To the members of the Hardwick Lab, both past and present – Heather Lamb, Tim Wang, Jason Huska, Zach Stolp, Eric Yau, Kyle Metz and Prerna Suri, I would like to thank each and every one for all the help you have given me. To Heather, I am especially grateful for your help in the laboratory. She have taught me not only how to think scientifically but also how to handle life's pressures. I also learned from Heather how to make time and set goals one at a time. I thank Tim for helping me with experiments and educating me in concepts essential to the lab. To Jason, Zach and Prerna, I am grateful

for the positive feedback on my “scientific outcomes” and for thoughtful contributions to my knowledge both in and out of the laboratory.

Out of the Laboratory, I have also gained support from the friends I have made in Baltimore and my family. I would like to thank all of my friends for making my two-year journey wonderful, memorable and fruitful. They have made the entire journey bearable by making me realize that there is life outside of the laboratory. To my family, especially my mom, I am especially grateful for the abundant unconditional support and for all of your efforts to understand my experiences throughout.

Lastly, I would like to thank all those who helped me to endure and persevere throughout these two years of bench science training, helping me to achieve beyond my expectations. From these experiences, I have learned to not doubt myself and I will remember these achievements as encouragement in my future endeavors. This entire journey was a great learning experience in many regards.

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INTRODUCTION

Potassium channel tetramerization domain (KCTD) proteins are a group of 26 understudied proteins containing a conserved BTB/POZ Broad-complex, Tramtrack, Bric-abrac/Poxvirus and Zinc finger) domain implicated in protein-protein interactions (Liu, Xiang, & Sun, 2013). While some KCTD proteins are predicted to have different biological and biochemical functions, including transcriptional regulation and as adaptors for protein ubiquitination, the function of KCTD proteins is essentially unknown, and the mechanisms by which mutations in these genes contribute to human disease is even less clear. KCTD13 is 329 amino acids long and is encoded on chromosome 16 at the 16p11.2 locus, and has been recently implicated in many neuropsychiatric disorders such as Autism Spectrum Disorder (ASD) and Schizophrenia (Golzio et al., 2012). Though, a genetic link between KCTD13 and ASD has been reported, a causal role for KCTD13 is not yet firmly established. Furthermore, the molecular mechanisms of KCTD13, or any other KCTD family member, have not been elucidated.

KCTD Family of Proteins

The human KCTD family of proteins is a group of 26 proteins sharing sequence similarities with the cytoplasmic T1 domain of voltage-gated K+ channels (e.g. Kv1.3, Kv3.1). Sequence alignment of KCTD family proteins reveal a conserved N-terminal sequence with BTB domain and variable C-terminal region (Liu et al., 2013). The 26 family members can be grouped into 7 clades or subfamilies A-G based on sequence similarity within and outside the BTB domain. Clade A consists of KCTD15 and

KCTD1, Clade B has KCTD11, KCTD21 and KCTD6, Clade C contains TNFAIP1, KCTD13 and KCTD10, Clade D contains SHKBP1 and KCTD3, Clade E contains KCTD17, KCDT5 and KCTD2, Clade F has KCTD12, KCTD16 and KCTD8, and Clade G consists of BTBD10 and KCTD20. While most KCTD family members are group in clades, some members are isolated such as KCTD19, KCTD4, KCTD14, KCTD7, KCTD9 and KCTD18 (Skoblov et al., 2013). Despite sequence similarity with T1 domains of K+ channels, there is no compelling evidence that KCTDs act as channels or directly bind to T1 domains.

KCTD proteins do not function as K+ channels as originally expected based on their sequence homology with the T1 domain of K+ channels. However, a well-characterized function for a subset of BTB domains found in other unrelated protein families is as an adaptor for Cullin-3 (Skoblov et al., 2013). The BTB domain of KCTD proteins is a highly conserved motif of about 100 amino acids located at the N-terminus of each member of the family. Approximately 400 BTB domain-containing proteins from a number of different protein families are encoded by the human genome including proteins involved in transcription, oncogenesis, and ion transport (Liu et al., 2013). In general, the BTB domains of KCTD family proteins mediate homo- and possibly heteroligomerization through their direct protein-protein interaction, and can also mediate interactions with non-family member protein targets.

KCTD proteins as potential Cullin-3 Adaptors

BTB Domain

The BTB domain facilitates a wide range of cellular functions from transcriptional regulation to protein ubiquitination due to its interactions with other protein-protein interaction motifs (Stogios, Downs, Jauhal, Nandra, & Privé, 2005). Four families of BTB-domain containing proteins are recognized in eukaryotes: BTB-Zinc Finger (BTB-ZF), Skp1, ElonginC and T1 (Stogios et al., 2005). First identified through the crystal structure of the human protein promyelocytic leukemia zinc finger (PLZF), the BTB-domain was determined to be a homodimer with a prolate ellipsoid shape with a central scaffolding of a cluster of five alpha-helices flanked by three short beta-sheets (Ahmad, Engel, & Privé, 1998). Sequence and structural analysisof the monomer core, approximately 95 residues, revealed four conserved residues (His-48, Leu-52, Ser-56 and Tyr-88) while the dimer interface, which determines the specificity and stability of the dimer varies across the four sequence families (Ahmad et al., 1998).

BTB proteins have been reported to be important transcription regulators, as in the case of the BTB-Zinc Finger (BTB-ZF) proteins PLZF and BCL6 (Stogios et al., 2005). The BTB domain of PLZF is responsible for the homodimerization of the translocation partner of PLZF, retinoic acid receptor alpha (RAR alpha) necessary for the transforming potential of the protein complex and also recruits the transcriptional co-repressor SMRT resulting to the inhibition of the target genes of RAR alpha effectively blocking differentiation of myeloid progenitors (Perez-Torrado, Yamada, & Defossez, 2006). Similar to PLZF, the BTB domain of BCL6 is also involved in transcriptional regulation. BCL6, an anti-apoptotic transcription factor in germinal center B-cells

represses the transcription of p53 and p21, thus allowing the B-cells of the germinal centers to bypass apoptosis and proliferate (Stogios et al., 2005). Aside from the ability of BTB proteins to regulate transcription factors, BTB-domain-containing proteins found in ion channels, called T1 (tetramer) domains, are responsible for bringing the four channel subunits together to form an active K+ channel (Perez-Torrado et al., 2006). Meanwhile, a separate group of BTB domain-containing proteins serve to recruit target proteins for ubiquitination by the E3 ubiquitin ligase cullin-3 (Perez-Torrado et al., 2006).

Protein Ubiquitination through Cullin

Protein ubiquitination regulates important regulatory proteins involved in many crucial cellular processes such as the cell cycle, inflammatory response and antigen presentation through the delivery of ubiquitinated protein targets to the 26S proteasome (Pickart, 2001). The process of ubiquitination involves the activity of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase enzymes (E3) resulting to the ATP-dependent proteolysis of the ubiquitin-conjugated substrate protein by the 26S proteasome (Willems, Schwab, & Tyers, 2004). The 76-residue polypeptide ubiquitin (ub) is bound to a cysteine residue of the E1 enzyme in an ATP coupled reaction followed by the transfer of ub to the cysteine residue of the E2 enzyme finally leading to the crucial step mediated by E3 wherein the C-terminal glycine of ub is bound to the protein substrate in primary sequence elements referred to as degrons (Hochstrasser, 1996). Despite the wide variety of protein substrates recognized by E3 Enzymes, most E3s contain one of the two catalytic domains, a HECT or RING domain (Pickart, 2001). The HECT domains (homologous to Eg-AP C-

terminus) function as ubiquitin carriers themselves while RING (really interesting new gene) domains class of E3s do not have inherent catalytic activity but rather use their Zn-binding motif to recruit and direct an E2 enzyme towards the substrate (Willems et al., 2004). An archetypal example of a RING domain E3 is the SCF-like (Skp1-Cullin1-F-box) complexes facilitated by the protein scaffold Cullin.

Cullins are globular proteins characterized by the presence of a C-terminal domain called the cullin-homology domain and a series of N-terminal repeats called cullin repeats (Petroski & Deshaies, 2005). Cullins form a complex with other proteins that together exhibit E3 activity and are referred to as Cullin-RING ligases (CRLs). Thus, cullins act as scaffolds for multi-subunit E3s. SCF, the model CRL,is the most studied of the many types of multi-subunit ub ligase through its interaction with CUL1 and provides the basis for many of the interactions facilitated by the seven different cullins expressed in the human proteome (CUL1, -2, -3, -4A, -4B, -5 and -7) (Petroski & Deshaies, 2005). Recently, BTB proteins such as the KCTD family have been identified in screens as interaction partners in the Cullin3 (SCF)-like E3 ubiquitin ligase facilitating recruitment of protein substrates to the Cul3 component of the SCF-like complex (Stogios et al., 2005)(Krek, 2003). The ability of BTB domain- containing proteins to bind the protein scaffold Cullin implicates proteins such as the KCTD family in protein ubiquitination. In addition, KCTD proteins, through their BTB domain can also interact with many protein substrates and as a result have many predicted functions of which transcriptional regulation and protein ubiquitination are the best characterized (Skoblov et al., 2013) (Stogios et al., 2005) (Liu et al., 2013).

Predicted Functions of KCTD Proteins

Via their BTB domains, KCTD proteins interact with many proteins including regulatory proteins needed for development and cell signaling (Zarellie & Dawid, 2013) (Ding et al., 2009) (Tong et al., 2014). Among the 26 current members of the family, KCTD15, a member of clade A, has been implicated in regulation of a transcription factor necessary for neural crest development. KCTD15 inhibits the transcription factor activating enhancer-binding protein 2 (AP-2), a critical protein in neural crest development. The inhibition of KCTD15 does not interfere with the dimerization of AP-2 and binding to its cognate site in the genome but rather binds specifically to the activation domain of AP-2, thus blocking the function of this critical factor in neural crest development (Zarelli & Dawid, 2013). In addition to KCTD15, another KCTD protein, KCTD1, has been implicated in the regulation of AP-2. KCTD1, a 257 amino acid protein expressed in the mammary gland, kidney, brain and ovary was originally characterized, as a transcriptional repressor acting through its BTB domain unaffected by histone deacetylase (HDAC) inhibitors. However the specific proteins regulated by KCTD1 at the time of its characterization was not determined (Ding et al., 2009) Recently, KCTD1 and transcription factor AP-2 alpha has been shown to interact and localize in nuclei, with KCTD1 overexpression repressing the transactivation of AP-2 alpha through its BTB domain suggesting that KCTD1 is a negative regulator of the transcription factor AP-2 (Ding et al., 2009). KCTD proteins have also been shown to regulate the transcription of regulatory transcription proteins in different organ systems such as the cardiovascular system. Mutations in KCTD10 result in changes in the expression of artioventricular markers, whereas wild type KCTD10 binds T-box

transcription factor Tbx5, a crucial factor in the formation of cardiac heart chambers, suppressing its activity (Tong et al., 2014). Aside from transcription regulation of factors necessary for development, KCTD proteins have also been shown to regulate important signaling molecules such as nuclear factor kappa B (NF-kB). Tumor necrosis factor alpha-induced protein 1 (TNFAIP1), another member of the KCTD family bound to KCTD10, has been shown to inhibit the transcriptional activities of NF-kB implicating KCTD10 in changes in the expression of target genes downstream of the immune response, inflammation and tumor cellular growth (Hu et al., 2012). In addition to transcriptional regulation, the BTB domain of KCTD proteins allows interaction with cullin-3 implicating a role of KCTDs in protein ubiquitination.

TNFAIP1, aside from binding KCTD10 and repressing the transcriptional activity of NF-KB, also promotes the degradation of its binding partner KCTD10 through protein ubiquitination (Hu et al., 2012). Acting through its BTB domain, TNFAIP1 binds to cullin-3 and promotes the degradation of KCTD10 (Hu et al., 2012). KCTD5, a cytosolic protein up regulated in post-transcriptionally in peripheral blood lymphocytes, also functions as a ligase for Cullin-3. Co-immunoprecipitation of Cullin-3 with expressed KCTD5 reveal binding of the proteins and ubiquitination of protein by the cullin-3/KCTD5 complex, suggesting the role of KCTD5 in recruiting protein substrates in protein ubiquitination. (Bayamon et al., 2008) KCTD1, on the other hand, mediates the ubiquitinaton of B-catenin, a factor along with Wnt, crucial in embryonic development and patterning (X. Li et al., 2014). Through the casein kinase 1 and glycogen synthase kinase-3beta mediated phosphorylation and enhancement by the E3 ubiqtuin ligase B-transducin repeat-containing protein, KCTD1 mediates the degradation of B-catenin

suggesting the role of KCTD1 in suppressing the canonical Wnt signaling (X. Li et al., 2014). The ability of KCTD proteins to interact with numerous proteins through their BTB domain has suggested a role of KCTDs in performing a variety of cellular processes. Thus, dysfunctions in their ability to bind substrates effectively might affect crucial cellular processes driving the emergence of human diseases.

KCTD proteins in Human Diseases

Current research, although sparse, implicates the KCTD family of proteins in a wide range of human diseases such as cancers, metabolism disorders and neurological diseases.

Cancers

Many genes and proteins have been implicated in cancer including KCTD family of proteins. KCTD12, first identified in the cochlea, has been identified in colorectal cancers (L. Li et al, 2016). Research suggests the involvement of KCTD12 in the regulation of colorectal cancer cell stemness where silencing of KCTD12 results to enhancement of stemness while overexpression leads to the repression of stemness. Molecularly, KCTD12 has been shown to suppress colorectal cancer stemness markers CD44, CD133 and CD29 through the inhibition of the metabolic pathway ERK (L. Li et al., 2016). The findings imply a potentially causal role for KCTD12 to colorectal cancers and the proposed use of KCTD12 as a prognostic marker for colorectal cancers (L. Li et al., 2016). KCTD12 has also been correlated to outcomes in the development and

diagnosis of gastrointestinal tumors (Hasegawa et al., 2013). Analysis of patient samples with gastrointestinal tumors reveals a higher 5-year survival recurrence-free survival rates in patients expressing KCTD12 compared to those who do not, consistent with a positive effect of the protein in suppressing tumors (Hasegawa et al., 2013). KCTD12 is the only KCTD protein suggested as a prognostic marker in gastrointestinal stromal tumors, however expression of KCTD10 in patient tumors has also been shown to have favorable clinical prognosis (Hasegawa et al., 2013) (Kubota et al., 2010). Immunohistochemistry of patient samples with gastrointestinal stromal tumors reveals a high disease-free survival rate in patients expressing KCTD10-positive tumors compared to those that are negative suggesting a tumor suppressive function of the protein. The results, similar to those of KCTD12, implicate KCTD10 in the suppression of tumors and a potential role for the protein as a novel prognostic biomarker in gastrointestinal stromal tumors (Kubota et al., 2013). Aside from cancers in the gastrointestinal system, KCTD proteins have also been implicated in cancers in other organ systems such as the nervous system. KCTD11 has been implicated in medullablastoma. Deletions of KCTD11 in chromosome 17p13.2, the gene most frequently lost in human medullablastoma, (Correale et al., 2011) reveal its role as tumor suppression in animal models. KCTD11 has been postulated to repress tumor formation through the ubiquitin-dependent degradation of histone deactylase HDAC1 thereby preventing the transcriptional activity of the hedgehog signaling pathway and limiting the oncogene (De Smaele et al., 2004). Further implicating its role in tumor suppression, deletion of the gene results in the deregulation of hedgehog signaling and formation of medullablastoma (Di Marcotullio et al., 2004), although controversies remain. More recently, other members of the KCTD family have been

implicated in the regulation of the Hedgehog signaling and hence the formation of medullablastoma. KCTD21 and KCTD6 have been both shown to bind cullin-3 thorough their BTB domains and ubiquitinate the essential regulator HDAC1 implicating both proteins, like KCTD11, in the regulation of the Hedgehog pathway (De Smaele et al., 2004). The involvement of KCTD proteins in cancers, whether gastrointestinal or neurological, has many scientific and clinical implications however the exact molecular mechanisms involved have not been clearly elucidated. Thus, a more thorough study of KCTDs including other members of the family is necessary to draw stronger conclusions regarding the role of KCTDs in cancer. Nevertheless, KCTD proteins have also been implicated in human diseases involving metabolism and other notable diseases.

Metabolism and Other Diseases

The role of KCTDs in metabolism and other human diseases has been a fairly novel and understudied field compared to other diseases such as cancers and neurological diseases. However, a couple of studies have implicated KCTD proteins in disorders of metabolism and other human diseases. KCTD10, apart from its implicated role in the suppression of gastrointestinal stromal tumors, has also been implicated in the modulation of HDL-cholesterol concentrations (Junyent et al., 2009). Genome-wide associations studies have linked KCTD10 SNPs (Single Nucleotide Polymorphism) of (V260VT --- c? and i5642G---C?) with lower HDL-cholesterol concentrations compared to carriers with minor alleles suggesting a potential role for the protein in lowering HDL-cholesterol concentrations in a population with high carbohydrate intakes. (Junyent et al., 2009) Genetic variants of KCTD15, on the other hand, have been shown to be associated

with fatness traits in chickens. Variations in diets have also shown differences in the expression levels of KCTD15, with fasted and re-fed chickens having markedly decreased levels of KCTD15 in the hypothalamus and liver while increased levels of the protein were seen in the adipose tissues of chickens fed with high-fat diet (S. S. Liang et al., 2015). The results suggest the role of KCTD15 in metabolism and a potential role in the regulation of obesity (Liang et al., 2015). KCTD proteins, aside from metabolism, have been implicated in other roles such as liver injury and Scalp-Ear-Nipple (SEN) syndrome (T. Chen et al., 2013) (Maneros et al., 2013). Liver injury, through infection, is fairly common although the exacerbation of a liver injury through immune activation is uncommon. KCTD9 has been recently shown to be involved in immune activation and increased liver damage in individuals infected with Hepatitis B virus (T. Chen et al., 2013). KCTD9 is highly expressed in the peripheral and hepatic NK cells of Hepatitis B Virus-induced acute-on chronic liver failure (HBV-ACLF) blood and liver samples compared to those samples with mild Chronic Hepatitis B (T. Chen et al., 2013). The upregulation of KCTD9 was also shown to correlate with the severity of liver injury, implicating the protein in contributing to the liver injury induced by hepatitis B (T. Chen et al., 2013). Mechanistically, KCTD9 increases CD69 expression, cytotoxicity and IFN gamma expression implicating the protein in the activation of NK cells. (T. Chen et al., 2013) Mutations in KCTD1, on the other hand, have been implicated in the development of SEN (Maneros et al., 2013). SEN is a rare, autosomal-dominant disorder characterizes by skin lesions of the scalp, anomalies of ears, digits and nails and abnormalities in the breast. Linkage studies reveal the connection between mutations in KCTD1 and SEN implicating its role in the disorder. KCTD1, functioning as an inhibitor of the

transactivation of the transcription factor AP-2 a, is an important transcriptional regulator during ectodermal development. Thus, mutations in KCTD1 prevent appropriate regulation during development contributing to the clinical manifestation of SEN in patients with KCTD1 mutations (Marneros et al., 2013). Despite the scarce literature on the role of KCTD proteins in metabolism disorders and other human diseases, the wide range of disorders implicating KCTDs suggest its diverse and important role in cellular processes.

Neurological diseases

Among the many human diseases involving KCTD proteins, the link to neurological diseases is much more compelling, with KCTD7 being the only KCTD causally linked to neurological conditions such as epilepsy, dystonia, schizophrenia and autism. Mutations in KCTD7 have been strongly implicated in progressive myoclonus epilepsies (EPM3), also known as CLN14 (neuronal ceroid lipofuscinosis 14), an autosomal recessive disorder characterized by generalized epilepsies, myoclonus, tonic-clonic seizures and progressive neurological deterioration. Clinically, EPM3 with KCTD7 mutations have a mean age of presentation at 16-18 months with psychomotor decline evident 0-22 months after the onset of seizures (Kousi et al., 2012)(Metz and Hardwick, unpublished). Located on human chromosome 7q11.21, mutations in this 31-kDa protein involving a missense change Arg184Cys has been shown in individuals with EPM3 mapped with a recessive pattern (Staropoli et al., 2013). This mutation has been postulated to result to the failure of KCTD7 to interact with cullin-3, thereby possibly leading to the build up of proteins and eventual cytotoxicity (Staropoli et al., 2012). Loss

of KCTD7 function through mutations, in addition to the postulated cytotoxicity, has also been associated with depolarized resting membrane potential and increased excitability of neurons, thus increasing the susceptibility for epileptic seizures while overexpression leads to the opposite phenotype (Kousi et al., 2012). Other mutations also lead to EPM3. The first report linking KCTD7 to epilepsy was a familial C-to-T mutation in exon 2 of KCTD7 resulting to the change from an arginine codon, CGA to a stop codon thereby truncating the protein also results to mapping of the EPM3 phenotype (Van Bogaert et al., 2007). The clinical phenotype expressed with KCTD7 mutations is consistent with its protein expression pattern in mice. KCTD7 is expressed predominantly in the brain, specifically in the cortical regions, in the granular and pyramidal cell layers of the hippocampus and in the cerebellar purkinje cells (Farhan et al., 2014). In addition to the current literature linking KCTD7 to EPM3, our lab has found that KCTD7 co-localizes in cells with known autophagy markers, suggesting a role for KCTD7 in autophagy, with further implications for the disease pathogenesis of EPM3 (Metz et al., unpublished). In addition to KCTD7, another KCTD protein, KCTD17, has been recently implicated in neurological diseases involving movement disorders. Linkage analysis and whole-exome sequencing of KCTD17 revealed a c.434 G>A p. (Arg145His) mutation in a familial pedigree of individuals with myoclonus-dystonia, a rare movement disorder characterized by combination of non-epileptic myoclonic jerks and dystonia. Despite the identification of the mutation and its linkage to myoclonus-dystonia, the characterization of the protein and functional analysis has yet to be determined for KCTD17 (Mencacci et al., 2015).

KCTD13

KCTD13, a 329 amino acid BTB-containing protein, has been recently implicated in neurological diseases (Lin et al., 2015)(Golzio et al., 2012). Located on human chromosome 16p11.2, a region associated with neurological diseases, KCTD13 copy number variations (CNVs) were associated with the emergence of phenotypes consistent with Autism Spectrum Disorders (ASDs) and schizophrenia (Lin et al., 2015) (Golzio et al., 2012). However, a unique causal role for KCTD13 in schizophrenia has not been established.

Autism spectrum disorder (ASD), a neurodevelopmental disorder, is characterized by varying degrees of difficulties in three areas: social interaction, verbal and nonverbal communication and repetitive behaviors. (Autism Speaks, 2016) Clinical diagnosis of ASD occurs at a very young age with clinical criteria emerging between 2 and 3 years of age (NIH, 2016). Approximately, 1 in 68 American children are diagnosed with ASD with a higher prevalence in boys compared to girls, with an estimated 1 out 42 boys and 1 in 189 girls diagnosed in the United States (Autism Speaks, 2016). Recent estimates suggest over 3 million individuals in the U.S. and tens of millions worldwide are affected by ASD (Autism Speaks, 2016). Despite the prevalence of the disorder and stringent clinical diagnoses, the cause of autism has not been elucidated although a combination of environmental risk, risk genes and even the immune system influencing early brain development have been implicated (Autism Speaks, 2016). Currently, studies have reported a correlation between 16p11.2 CNVs and ASD, implicating the loci in development of the disorder (Hanson et al., 2013) (Ripke et al., 2014).

Individuals with 16p11.2 deletions have a wide range of neurodevelopmental outcomes and have higher rates of psychiatric and developmental disorders compared with familial non-carrier individuals, suggesting a link between these deletions and the neurodevelopmental outcomes (Hanson et al., 2013) (Kumar et al., 2008). However, an understanding of the pathways disrupted by these 16p11.2 CNVs is lacking. Analysis of the human brain transcriptome from exon microarray data and protein network mapping however, reveals protein-protein interactions in distinct brain regions at distinct times during development (Lin et al., 2015). Spatiotemporal analysis of the brain transcriptome reveals enrichment in 16p11.2-interacting proteins in the late mid-fetal and childhood periods in the cortex, periods that are consistent with the neurodevelopmental progression of ASD (Lin et al., 2015). Further analysis has also identified de novo ASD mutations in the 16p11.2 locus that are highly enriched in spatiotemporal networks, including KCTD13 as one of the strongest candidates and a major contributor to the neuropsychiatric phenotypes observed in the 16p11.2 CNVs (Golzio et al., 2012).

Close analysis of the 16p11.2, containing 29 genes, reveals that among these 29, KCTD13 is the sole gene responsible for head size phenotypes (Golzio et al., 2012). Using Zebrafish as a model, Glozio et al noted phenotypic differences in headsizes of zebrafish with varying expression of KCTD13. Overexpression of human KCTD13 mRNA transcripts in zebrafish resulted to microcephaly, while a splice bocking morpholino against exon 3 of KCTD13 resulted to a macrocephalic phenotype (Golzio et al., 2012). The observed phenotypes have been postulated to arise from the differences in cell proliferation and death of neuronal progenitors in the developing brain. In the case of microcephaly, histone H3 and TUNEL staining reveals a decrease in neuronal progenitors

accompanied by an increase in apoptosis while the opposite is true for the case of macrocephaly (Golzio et al., 2012). The findings in zebrafish mirror the observed phenotypes with humans suggesting its dosage changes as a precursor for neurological disorders such as ASD. (Golzio et al., 2012) Furthermore, analysis of independent human subjects with ASD also concurs with the observations with CNVs suggesting a strong link between KCTD13 and ASD (Golzio et al., 2012).

Analysis of the protein networks of KCTD13 during development indicates physical interactions with seven proteins across distinct spatiotemporal intervals (Lin et al., 2015). KCTD13 physically interacts with PCNA-POLD2-TNFAIP1-KCTD10, suggesting a role of the protein in DNA replication in earlier time periods (Lin et al., 2015). KCTD13, also known as polymerase delta-interacting protein 1 (PDIP1), was first identified from a two-hybrid screen of a HepG2 cDNA library by using p50 as bait and a cDNA encoding a protein of 36 kDa as the interacting protein (Zhou et al., 2005). The initial findings suggest a direct interaction between KCTD13 (PDIP1) with the proliferating cell nuclear antigen (PCNA) and the p50 subunit of the polymerase delta (polD), further stimulating the activity of the PCNA-dependent polymerase delta activity (H. He, Tan, Downey, & So, 2001). The interaction observed also dictated the localization of KCTD13 in the nucleus, co-localizing with PCNA at replication foci (H. He et al., 2001). Sequence similarity between KCTD13 and the protein B12, an early response gene induced by tumor necrosis factor alpha, suggested the induction of the KCTD13 with TNF-alpha. Indeed, treatment with TNF-alpha and IL-6 resulted in an increase in KCTD13 levels in HepG2 cells, implicating the gene as a plausible link between cytokine activation and DNA replication. (H. He et al., 2001)KCTD13 was also

shown to form a complex with Cul3-TNFAIP1-KCTD10, further implicating KCTD13 in regulation and control of not only DNA replication but also of the cell cycle (Y. Chen et al., 2009a).

KCTD13, aside from its suggested role in cell cycle regulation, has also been implicated in the regulation of the small GTPase RhoA, a major regulator of actin cytoskeleton and cell migration. (Lin et al., 2015) Knockdown of Cul-3 in HeLa cells resulted in abnormal actin stress fibers and distorted cell morphology suggesting the role of Cul-3 in regulating actin cytoskeleton structure (Lin et al., 2015). RhoA, a direct target of the ubiquitin ligase complex, is presumed to be ubiquitinated by the Cul3/KCTD13 complex resulting to the degradation of RhoA (Lin et al., 2015). The Cul3-KCTD13-RhoA complex and regulation of RhoA levels was determined to be essential for migration of cultured cells and cell movement during *Xenopus* gastrulation (Y. Chen et al., 2009b). Thus, dosage changes in 16p11.2 CNVs might lead to changes in the regulation of the RhoA pathway, important in neuronal development including neurite outgrowth, axon path finding, neuronal migration and maintenance ultimately leading to impairments in neural development (Lin et al., 2015). Analysis of de novo mutations in Cul-3 p.Glu246Stop (E246X) and p.Arg546Stop (R536X) in individuals with ASD shows a disruption with binding to KCTD13 suggesting the specificity of the binding between KCTD13 and Cul-3 and a possible role of these mutations in deregulation of RhoA (Lin et al., 2015).Despite all the suggested roles and disease associations of KCTD13, a molecular understanding of its functions and roles in cellular processes has not been elucidated. The scarcity of studies regarding the exact nature of KCTD13 is lacking and therefore strong conclusions regarding the exact role of the protein is somewhat

inconclusive. Therefore, characterization of the protein is necessary to draw coherent conclusions regarding its potential role in cellular processes and ultimately its role in the development of neuropsychiatric disorders such as ASD.

AUTOPHAGY

Autophagy, derived from the Greek meaning “eating of self”, is a catabolic process important for maintenance of cell homeostasis, adaptive response to nutrient stresses and degradation of organelles and proteins (Glick, Barth, & Macleod, 2010). Autophagy occurs at a basal rate and is up-regulated in unfavorable conditions and stresses such as nutrient depravation, allowing macromolecules in the cytosol to be recycled back for reuse during starvation thus, serving as an adaptive response in changing cell dynamics (C. He & Klionsky, 2009). Characterized by the formation of double membrane vesicles, called autophagosomes, autophagy facilitates degradation of proteins, damaged organelles and even pathogens through the delivery of cargo in the autophagosome to the lysosome (C. He & Klionsky, 2009). First coined by Christian de Duve, the process was largely based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with glucagon (Glick et al., 2010). Despite the early observation of autophagy in mammalian cells, the molecular mechanism leading to autophagy has not been uncovered until recently through an understanding of autophagy in *S. cerevisiae* (Yang & Klionsky, 2010a). Molecular studies in *S. cerevisiae* led to the identification of 32 autophagy-related genes (ATG) genes, orchestrating the sophisticated molecular machinery of autophagy in yeast. Analysis of the ATG genes in yeast show mammalian homologs suggesting that

autophagy is a highly conserved pathway (C. He & Klionsky, 2009). Currently, there are three defined types of autophagy: macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA), all of which promote degradation of cytosolic contents at the lysosome (Glick et al., 2010). Macro-autophagy involves the formation of the autophagosome containing cytoplasmic contents such as intact organelles, proteins and portions of the cytoplasm. The autophagosome, along with its contents matures by fusion with and endosome and/or lysosome forming an autolysosome allowing the breakdown of autophagosome cargo (Yang & Klionsky, 2010a). Microautophagy, on the other hand, involves the direct engulfment of cytoplasm at the lysosomal surface, while CMA translocates unfolded, soluble proteins, directly across the membrane of the lysosome in complex with chaperone proteins recognized by lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A) (Glick et al., 2010). Despite the identification of three distinct types of autophagy, most studies have focused on macroautophagy and its role in human diseases; thereby the focus of this thesis is on macroautophagy and hereby referred to as autophagy throughout the document. Among the 32 ATG genes identified in yeast, a subset referred as the "core" molecular machinery is essential for autophagosome formation and hence autophagy. The core molecular machinery are: the Atg1/Unc-51-like kinase (ULK) kinase, the Class III phosphatidylinositol 3-kinase (PtdIns3K)/Vps34 complex I, and the Atg12 and Atg8/LC3 ubiquitin-like protein conjugation systems each responsible for distinct steps in the autophagic pathway (Yang & Klionsky, 2010a).

Molecular Machinery of Autophagy

The autophagic membrane biogeneis can be divided into a series of steps, initiation at the phagophore assembly site (PAS), elongation, expansion and completion of the autophagosome and autophagosome maturation via docking and fusion with endosomes and/or lysosomes, leading to the breakdown and degradation of autophagosome contents (Yang & Klionsky, 2010a). Each of the steps in the pathway involves the core molecular machinery and also other non-ATG genes.

Autophagy occurs at a low basal rate under normal conditions and is induced by environmental stresses such as nutrient depravation induces autophagy. (Glick et al., 2010) Studies in yeast reveal a central inhibitor of autophagy, the serine/threonine protein kinase TOR (target of rapamycin).TOR inhibition through starvation or treatment with rapamcyin leads to the activation of autophagy through the activation of the kinase activity of Atg1 (C. He & Klionsky, 2009). The activation of Atg1, in yeast, leads to a higher affinity binding to Atg13 and 17 leading to the formation of an Atg1-Atg13-Atg17 scaffold allowing for the recruitment of multiple Atg proteins to the PAS, initiating autophagy (Glick et al., 2010). Mammals, on the other hand, initiate autophagy similar to yeast through mammalian homologs of yeast ATG. Unc-51-like Kinase 1 (ULK1) and -2 (ULK), FIP200 (the focal adhesion kinase family-interacting protein of 200 kD) and mammalian Atg13 (mAtg13) are the mammalian homologs of Atg1, Atg17 and Atg13 respectively (C. He & Klionsky, 2009). Upon inhibition of mTOR (the mammalian homolog of TOR) through starvation or rapamycin treatment, mTORC1 dissociates from the ULK1-FIP200-Atg13 complex leading to the induction of autophagy (Yang & Klionsky, 2010a). The mTORC, similar to the yeast models, localizes to the phagophore

upon starvation. However, unlike yeast the mammalian PAS is not present (or has not yet been discovered). The origins of the double-membrane autophagosomes in mammalian cells are still highly debated. Studies suggest that autophagosome membranes grow from the endoplasmic reticulum and/or the trans-Golgi and endosomes through budding (Glick et al., 2010), while others suggest the addition of new membranes to the growing autophagosome nucleated sites by vesicle fusion (C. He & Klionsky, 2009). Despite studies attempting to locate the origins of these double membrane vesicles, a clear conclusion has not been reached.

Once the induction of autophagy occurs, either through starvation or rapamycin treatment, the nucleation and assembly of the initial phagophore membrane ensues. Nucleation and assembly requires the Class III PtdIns3K complex composed of Vps34 (Vacuolar protein sorting 34), Vps15 (p150 in mammalian cells), Atg14 (Barkor or mAtg14 in mammalian cells) and Atg6/Vps30 (Beclin 1 in mammalian cells) (C. He & Klionsky, 2009). In yeast cells, Vps34 is essential for generating phosphatidylinositol (3)-phosphate (PtdIns(3)P) at the PAS, allowing the recruitment of Atg proteins necessary for the elongation and expansion of the phagophore such as Atg18, Atg20, Atg21, and Atg24 (Yang & Klionsky, 2010a). In mammalian cells, the PtdIns3K complex also produces PtdIns3P targeting the recruitment of mammalian homologs of yeast Atg proteins necessary for elongation and expansion of the phagophore.

The PtdIns3K complex along with the Atg proteins recruits two overlapping ubiquitin-like conjugation systems, Atg12-Atg5-Atg16 and Atg8-LC3 (phosphatidylethanolamine) to the phagophore initiating its elongation and expansion (Glick et al., 2010). In both yeast and mammals, Atg12 is conjugated to Atg5 in a

reaction that requires the E1 and E2-like enzymes Atg7 and Atg10. The Atg12-Atg5 conjugate then interacts with Atg16L resulting to its oligomerization to a large multimeric complex referred to as the Atg16L complex (Yang & Klionsky, 2010a). On the other hand, the Atg8/LC3 conjugate is cleaved by Atg4 at its C-terminus, generating the cytosolic LC3-I with a C-terminal glycine residue, which is then conjugated to a PE (phosphatidylethanolamine) through a reaction mediated by Atg7 and the E2-like enzyme Atg3 to form LC3II (C. He & Klionsky, 2009). LC3-II, the lipidated form of LC3 is attached to both faces of the phagophore membrane although, as the process of phagophore maturation occurs, it is removed from the outer membrane of the autophagosome following the fusion of the autophagosome with late endosome/lysosome (Glick et al., 2010). Upon completion, the autophagsome docks and fuses to late endosomes/lysosomes resulting to the degradation of autophagosome contents.

Fusion of the mature autophagosome, in mammalian cells, requires the lysosomal membrane LAMP-2 and the small GTPase Rab7 (Yang & Klionsky, 2010a). In comparison, the machinery facilitating the fusion of the autophagosome and the late endosome/lysosome are more detailed. In yeast, the GTPase Ypt7 (homolog of Rab7), Sec18, the SNARE proteins Vam3, Vam7, Vti1 and Ykt6, class C Vps/HOPS complex proteins and CxC1 and Mon1 are required to facilitate the docking and fusion event (C. He & Klionsky, 2009). Fusion of the autophagosome and the lysosome results to the degradation of the inner vesicle of the autophagosome along with its contents. The degradation of contents is dependent on the action of a variety of lysosomal/vacuolar acid hydrolases including proteinases A and B, lipase Atg15 in yeast and cathepsins B, D and L in mammalian cells (Glick et al., 2010). The macromolecules produced from the

degradation of autophagic contents are transported back to the cytoplasm where molecules such as amino acids are reused for protein synthesis and maintenance of cellular functions under nutrient starvation (C. He & Klionsky, 2009). While autophagy is thought to only degrade bulk cytoplasmic contents, current studies suggest the existence of selective autophagy wherein specific cargos are recognized and degraded through interactions with specific receptor proteins (Glick et al., 2010).

Cargo recognition and selectivity in selective autophagy involves specific proteins identifying distinct cargo in both yeast and mammalian cells. In yeast, the protein prApe1 is targeted to the vacuole generating the mature enzyme Ape1 through the Cvt (cytoplasm to vacuole targeting) pathway. The protein prApe1 contains a vacuolar-targeting signal recognized by the receptor protein Atg19, together the Atg19-prApe1 complex is bound by the adaptor protein Atg11 delivering the complex to the PAS where Atg19 interacts with key components of the vesicle-forming machinery (C. He & Klionsky, 2009). In mammalian cells, numerous studies have suggested the clearance of cytosolic ubiquitinated substrates or aggregate prone proteins through selective autophagy. The mammalian protein p62/sequestome 1 (SQSTM1) has been shown to directly bind both poly- or monoubiquitin via its ubiquitin-associated (UBA) domain and LC3, linking ubiquitinated cargos for autophagic degradation (Glick et al., 2010). Mitophagy, the selective autophagic degradation of the mitochondria, has also been a focus of studies suggesting the existence of selective autophagy. While the process of mitophagy in mammalian cells has not clearly been elucidated, clues from yeast studies have surfaced. The first mitophagy protein identified was the yeast protein Uth1p required for mitochondrial clearance by autophagy although the interaction between the protein and

the autophagosome has not yet been elucidated (He et al., 2010). In addition, mammalian homologs of the yeast protein Uth1 have not been discovered. Other proteins implicated in mitophagy are the mitochondria anchored protein Atg32, BNIP3L a protein involved in mitochondrial clearance in differentiating red blood cells, Ulk-1 and Parkin an E3 ubiquitin ligase located at the outer mitochondrial membrane (Glick et al., 2010). Despite evidence of selective autophagy, a clear understanding of the molecular pathways involved in the process is still lacking. However, given the importance of autophagy in the maintenance of cell homeostasis and function, defects in autophagy have been implicated in the genesis of numerous human diseases.

Autophagy and KCTD proteins

Our interest in in the KCTD family of proteins arises from data obtained from yeast genetic screens revealing that secondary mutations in *WHI2* (whiskey-2, "wee-2") affecting nutrient responses and stress-induced cell death commonly arise following deletion of specific genes (e.g. the conserved mitochondrial fission factor Fis1) in *Saccharomyces cerevisiae* (Cheng et al. 2008) (Teng et al., 2013). *WHI2* is a poorly characterized stress-response gene. Our lab identified *WHI2 as a* regulator of nutrient-sensing and cell survival (Teng et al., 2013). Following amino acid withdrawal, a known autophagy stimulus, *WHI2* is required to reduce TOR kinase activity and to slow cell growth and induce autophagy (Teng et al., 2013)(Teng, Cheng and Hardwick, unpublished). Thereby, suggesting that the Whi2 protein may be a new negative upstream regulator of TOR and implicating Whi2 in the autophagy pathway (Teng and Hardwick, unpublished). Bionformatics tools revealed human homologs for the once thought fungi-

specific yeast gene WHI2, identifying the KCTD family of proteins as human homologs (Teng et al., 2013). Based on the results from yeast homology, our goal is to characterize KCTD13 by examining its subcellular localization and thereby, determining whether the protein plays a role in the autophagy pathway.

METHODS

Plasmid Constructs

N-terminal HA (hemaglutinin)-tagged full length (FL) and BTB domain (FL: 360 AA; BTB:141 AA) of human KCTD13 were generated by Master's student Feria Ladha in the laboratory (Ladha, 2014). C-terminal HA-tagged full-length KCTD13 was generated by PCR amplification from the HA-KCTD13FL plasmid and inserted into an HA expression vector pDB59 using infusion cloning technique (Clontech). GFP-tagged human KCTD13 FL, BTB, and C-terminus (FL: 360 AA, BTB: 141 AA, C: 120AA) were created similarly but were inserted to GFP expression vector pHML8 (5'TGC TCG AGA TGT TCT GAT GAG AAG TTC GTA GAC GAC CCC 3') (5'GTT CGA ACC ACT GGT TCA GTG CTT GAA GAC AAT 3'). All constructs were sequenced confirmed and verified. RFP-Rab5 was purchased from Clonetech/JHU HIT Center, GFP-Rab7, RFP-LC3, GFP-LC3B, RFP-Rab11A, RFP-Rab11B, GFP-Atg12 and Myc-Cul-3 were obtained as gifts from Dr. Isabelle Coppens, Dr. Anne Hamacher-Brady, Dr. James Wohlschlegel and other labs.

Cell Culture and Mitochondrial Uncoupling

Henrietta Lacks (HeLa) and African Green monkey kidney cells (COS-7) were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 1% penstrep. Cell cultures were incubated at 37C, 5%

CO2. Mitochondrial uncoupling was induced by incubating cultures with 0.5 mL of 25 mM CCCP (carbonyl cyanide m-chlorophenyl hydrazine) for 4 hours.

Transfection and Immunostaining

Cells were plated on coverslips in a 24-well plate 24 hours before transfection. Cells were transfected with a total of 250 ng of DNA mixed with 0.75 μL of XtremeGene (Roche) and 25 μL of Optimem per well. After 24 hours post transfection, cells were washed with cold PBS and were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed three times with cold PBS and permeabilized with 0.2% Triton-X100 for 5 minutes. Cells were washed three times with cold PBS and were blocked with 2% goat serum for 30 minutes at room temperature and then treated with primary antibody (1:1000 dilution for all antibodies except Anti-GFP diluted at 1:2000) for 1 hour at room temperature. After 1 hour, cells were washed again thrice with cold PBS and were blocked again with 2% goat serum for 30 minutes before the addition of the secondary antibody (1:1000 dilution) for 1 hour at room temperature. Cells were washed with cold PBS thrice and were treated with Phalloidin (for actin staining conditions) for 30 minutes or directly treated with DAPI (1:4000 dilution) for 10 minutes at room temperature and then mounted onto slides using Gold Anti-fade Reagent (Invitrogen). Slides were left at 4 °C overnight before acquiring images. For mitotracker treated cells, mitotracker (1:4000) dilution is added for 10 minutes then removed for recovery in fresh media before immunostaining.

Fluorescence Microscopy and Data Analysis

A Zeiss M2 fluorescence microscope was used to acquire images at 100x objectiveand analyzed using Volocity analysis Software (PerkinElmer).

Lysate Collection and Western Blot Analysis

Mouse lysates were obtained from male and female 5-week 129 mice and suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris PH 8.0). COS-7 cells were transfected with DNA plasmids and cell lysates were collected 24 hours later and suspended in 1x sample buffer. Lysates were vortexed for 10 minutes and boiled for 5 minutes at 100xC. Lysates were loaded onto 12% SDS-PAGE gel, transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and detected with enhanced chemiluminesence (ECL) Western blotting detection reagents (GE Healthcare Life Sciences).

RESULTS

Subcellular Localization of KCTD13 is Cytoplasmic

To begin to characterize the KCTD13 protein, expression constructs were generated to overexpress epitope-tagged KCTD13 in cultured cell lines. Full length KCTD13, a 329 amino acid protein (denoted as KCTD13FL throughout the document), was epitope-tagged with HA or GFP at its N-terminus (Figure 2A). BTB domains are known to be protein-protein interaction domains (Petroski & Deshaies, 2005), and the C-terminal regions of KCTD proteins are unique to this family. To distinguish the subcellular targeting information present in each half of KCTD13 separately, N-terminal HA-tagged and GFP-tagged versions of both the BTB and the C-terminal regions were tested in parallel (Figure 2B and 2C). The N-terminal fragment of KCTD13, herein referred to as KCTD13BTB, contains amino acids 1-141, encompassing the BTB domain (Figure 2B). The C-terminal construct, referred to as KCTD13C, contains amino acids 210-329 (Figure 2C).

Expressed KCTD13FL leads to the formation of three distinct cytoplasmic structures in HeLa cells: small punctate, punctate and vesicle-like (Figure 3). Both HA- and GFP-tagged KCTD13FL predominantly resulted in the small punctate morphology in 60-70% of transfected cells (Figure 3A). Approximately 20-30% of cells express puncta-like structures ranging in sizes from 0.5-2μm (Figure 3B), which is around the reported size of an autophagosome (0.5-1.5 μm) (Pattingre, Espert, Biard-Piechaczyk, & Codogno, 2008). In approximately 10% of the cells, KCTD13 formed large vesicle-like structures (Figure 3C and 3D). Quantification of cell morphologies between the HA-and GFP-tagged revealedno differences between the two constructs (Figure 3D).

The overexpression of KCTD13BTB leads to the formation of two morphologies in HeLa cells: diffuse cytoplasmic and vesicle-like structures (Figure 4). Approximately 90% of the cells expressing HA- or GFP-KCTD13BTB exhibit diffuse cytoplasmic staining but can also concentrate in the nucleus. The nuclear localization cannot be explained by trapping of the GFP moiety in the nucleus, as HA-tagged KCTD13BTB exhibited similar concentration in the nucleus in a portion of the cells (Figure 4A and 4C). In the remaining 10% of transfected cells, KCTD13BTB adopted a distinct vesicle-like morphology (Figure 4B and 4C). The vesicle-like morphology of overexpressed KCTD13BTB is often concentrated in areas surrounding the nucleus. Similar to expressed KCTD13 FL, the HA- and GFP-tagged KCTD13BTB constructs did not differ in their subcellular localization patterns (Figure 4C).

In contrast to full-length and BTB, KCTD13C can form aggregate structures in approximately half of the transfected HeLa cells (Figure 5A-C). Similar to the expression of KCTD13FL and BTB, KCTD13C can be diffusely cytoplasmic in HeLa cells (Figure 5A). In addition, KCTD13C can also form aggregate structures in half of the cells quantified (Figure 5B). Similar morphologies of KCTD13C were observed for both the HA- and GFP-tagged constructs (Figure 5C) The presence of distinct morphologies such as the punctate-like and vesicle-like structures formed by KCTD13FL, the vesicle-like structures formed by KCTD13BTB, and the aggregate structures formed by KCTD13C may suggest a progression from the cytoplasmic structures to these distinct structures, possibly a role of KCTD13 in a cytoplasmic process not previously considered.

To begin to identify the structures labeled by expressed KCTD13, co-localization studies were performed. KCTD13 has been implicated in the protein regulation of RhoA, a protein necessary for cytoskeletal rearrangements (Y. Chen et al., 2009). Although KCTD13FL does not form F-actin-like structures when transfected cells were co-stained with phalloidin to detect F-actin (Figure 6A). However, many of the cytoplasmic KCTD13 puncta were aligned adjacent or associated with F-actin filaments, either because of direct association or because KCTD13 associates with other structures that interact with actin filaments (Figure 6A, insets), consistent with the purported role of KCTD13 in cytoskeletal re-arrangement (Y. Chen et al., 2009). In contrast, anti-calnexin-labeled endoplasmic reticulum (Figure 6B) and anti-Giantin-labeled Golgi organelles involved in the endomembrane system and more recently hypothesized to contribute to autophagosome biogenesis (Glick et al., 2010), do not appear to co-localize with KCTD13FL (Figure 6B and 6C).

To determine if KCTD13 localizes to mitochondrial organelle, which are critical for the generation of metabolic energy, mitochondria were labeled with the fluorescent dye, MitoTracker. GFP-KCTD13FL does not directly co-localize with mitochondria, but instead commonly localizes to structures that appear to be associated with mitochondria (Figure 7A). However, cytoplasmic vesicle-like puncta observed with expressed GFP-KCTD13BTB strongly co-localize with MitoTracker (Figure 7B, white arrowheads). Furthermore, the aggregate-like morphologies observed with GFP-KCTD13C also co-localizes with MitoTracker (Figure 7C, white arrowheads). This finding raises the question whether KCTD13 performs a function involving the mitochondria.

Expressed KCTD13 Localizes with Expressed Cullin-3 and Mitochondria

KCTD13 was predicted to function as an adaptor for the E3 ubiquitin ligase Cul-3, a known function of BTB domains in other gene families (Liu et al., 2013). While several other KCTDs were recently reported not to interact with cullin-3 (Smaldone et al., 2015), there have not been any studies testing the association of KCTD13 with cullin-3, aside from predictive binding using the closest homolog of KCTD13, KCTD10 (Stogios et al., 2005). Expressed N-terminal 6-Myc-tagged Cul-3 results in diffuse cytoplasmic localization in HeLa cells (Figure 8A). However, co-expression of HA-KCTD13FL dramatically recruits Cul-3 to adopt the punctate structures characteristic of KCTD13. Furthermore, co-expression of Myc-Cul-3 increases the percent of cells with HA-KCTD13FL-labeled vesicle-like structures in the cytoplasm wherein both Cul-3 and KCTD13FL co-localizes (Figure 8B, compare to Figure 3C). In contrast, the vesicle-like structures formed by HA-KCTD13BTB did not co-localize with Myc-Cul-3 in the cytoplasm with both proteins localizing diffusely in the cytoplasm (Figure 8C). However, HA-KCTD13BTB forms vesicle like structures inside the nucleus, but these lack Myc-Cul-3 (Figure 8C, compare to Figure 4B). These nuclear structures are different from the smooth, diffuse localization of HA-KCTD13BTB in the nucleus without Cul-3 (Figure 8C, compare to Figure 4A). HA-tagged versions of KCTD13C also co-localize with Myc-Cul-3 in aggregate-like structures near the nucleus, reminiscent of the aggregate-like structures observed with expressed KCTD13C alone (Figure 8D, compare to Figure 5B). The co-localization of HA-KCTD13 with Myc-Cul-3 is consistent with a direct association of Cul-3 and KCTD13, as predicted for BTB domain-containing proteins, pending confirmation. The Cullin-RING-based BCR (BTB-CUL3-RBX1) E3 protein

ligase complex mediates the ubiquitination and subsequent degradation of target proteins such as the mitochondrial proteins (Petroski & Deshaies, 2005) .

To test whether KCTD13 might participate in the E3 protein ligase complex involved in degrading dysfunctional mitochondria, HeLa cells were treated with CCCP, a mitochondrial uncoupler, to induce mitochondrial damage. Whereas KCTD13FL only associated but did not co-localize prior to depolarization of mitochondria, treatment with CCCP results in a visually strong co-localization of HA-KCTD13 with mitochondria (Figure 9A, compare to Figure 7A). GFP-KCTD13BTB and GFP-KCTD13C structures continue to co-localize with mitochondria following CCCP treatment except more intensely (Figure 9C). Similar to the results with mitochondria, the co-localization between all KCTD13 constructs and Myc-Cul-3 intensifies after treatment with CCCP.

HA-KCTD13FL forms an increased number of vesicle-like structures in the cytoplasm wherein Myc-Cul-3 is recruited after CCCP treatment (Figure 10A). The structures are noticeably bigger and more aggregated compared to non-CCCP treated cells. (see Figure 8A). After CCCP treatment, HA-KCTD13BTB forms vesicle-like structures where Myc-Cul-3 co-localizes, distinct from the cytoplasmic co-localization observed in cells without CCCP treatment (Figure 10B, compare to Figure 8C). In addition, treatment with CCCP leads to the disappearance of the KCTD13BTB vesicle-like structures formed in the nucleus (see Figure 8B). HA-KCTD13 C, similar to cells not treated with CCCP, also co-localize with Myc-Cul-3 in aggregate-like structures surrounding the nucleus (Figure 10C). However treatment with CCCP increases the co-localization observed and forms more aggregate-like structures (Figure 10C, compare to Figure 8D).

Consistent with the increased localization between expressed KCTD13 and mitochondria and between expressed KCTD13 and Myc-cul-3 after CCCP treatment, visualization of all three proteins: KCTD13, mitochondria and Myc-Cul-3 revealed that both KCTD13 and Myc-Cul-3 co-localize on damaged mitochondrial organelles. Without CCCP treatment, the co-localization between HA-KCTD13FL and Myc-Cul-3 is retained (see Figure 8B), however co-localization of HA-KCTD13FL and Myc-Cul-3 with mitochondria is not observed (Figure 11A). Treatment with CCCP, however, reveals a strong co-localization between HA-KCTD13 FL, Myc-Cul-3 and the mitochondria using 4-color microscopy (Figure 11B) suggesting a possible role of KCTD13 in regulating mitochondrial homeostasis through association with Cul-3. Functioning as an E3 ligase, Cul-3, ubiquitinates damaged proteins for degradation through the proteasome although it has also been shown to be involved in protein degradation through the autophagy pathway (Petroski & Deshaies, 2005). More recently, Cul-3 was also reported to play a role in regulating the late steps of the endolysosomal pathway (Hubner & Peter, 2012). Taken together, the localization of expressed KCTD13 with Myc-Cul-3 and mitochondria after CCCP implicates a potential role of KCTD13 in mitochondrial homeostasis. Table 1 summarizes the sub-cellular co-localization of overexpressed KCTD13 (Table 1).

Expressed KCTD13 Localizes with Endosome Markers RAB5 and RAB7

To further elucidate the potential role of the KCTD13 in the degradation of damaged mitochondria, co-localization of KCTD13 with endosome markers was performed. Early endosome marker RFP-RAB5 was co-expressed with GFP-KCTD13 constructs. Co-expression of RFP-RAB5 and GFP-KCTD13FL predominantly forms in

KCTD13FL punctate-like structures, but they also both cluster together around the nucleus (Figure 12A). In contrast, neither GFP-KCTD13BTB nor GFP-KCTD13C co-localize with RFP-RAB5 (Figure12B and 12C). GFP-KCTD13BTB retains its diffuse cytoplasmic morphology and appears not to alter RFP-RAB5 (Figure 12B). However, An association with GFP-KCTD13C and RFP-RAB5 may occur as both are clustered near the nucleus (Figure 12C).

To further assess the involvement of KCTD13 in the endosome pathway, co-localization with late endosome marker RAB7 and the recycling endosome marker RAB11 were also performed. All three GFP-KCTD13 constructs co-localize strongly with expressed RFP-RAB7 (Figure 13). GFP-KCTD13FL co-localizes with late endosome marker RFP-RAB7, with a majority of the co-localization occurring close to the nucleus. Co-expression of RFP-RAB7 with GFP-KCTD13FL results in concentration of KCTD13FL puncta in areas surrounding the nucleus, only occurring with the presence of RFP-RAB7 and not any other marker (Figure 13, see Figure 12). Similarly, GFP-KCTD13BTB and C also co-localize with RFP-RAB7-labeled vesicle-like structures. GFP-KCTD13BTB, co-expressed with RFP-RAB7, results in a dramatic morphological change of GFP-KCTD13BTB from diffuse to vesicle-like structures near the nucleus (Figure 13B). Consistent with GFP-KCTD13FL and BTB, GFP-KCTD13C also co-localizes with vesicular RFP-RAB7 resulting to a dramatic morphological change of GFP-KCTD13C from diffuse to vesicle-like (Figure 13C).

In contrast to RAB5 and RAB7, GFP-RAB-11A and –B, which localize diffusely in the cytoplasm and with fine punctate near the nucleus, do not co-localize with KCTD13FL (Figure 15). The observed co-localization of KCTD13FL constructs with

both RFP-RAB5 and RFP-RAB7 suggests a potential role of KCTD13 in the endosomal pathway, perhaps through the reported association between Cul-3 and the early/late endosomal markers (Hubner & Peter, 2012). The observed co-localization of GFP-KCTD13FL, BTB and C with RFP-RAB7, implies that the protein functions in the late stages of the endosomal pathway.

Treatment with CCCP, however, changes the co-localization observed between the RFP-RAB5, RFP-RAB7 and GFP-KCTD13FL. While RAB5 and GFP-KCTD13FL co-localize without treatment, CCCP treatment abolishes the observed co-localization. With CCCP treatment, a visible increase in RFP-RAB5 vesicles is apparent, however it does not contribute to co-localization with GFP-KCTD13FL, as the protein appears to be more aggregated and separated from the vesicular RFP-RAB5 (Figure 15A). The disappearance of co-localization with GPF-KCTD13FL and RFP-RAB5 with CCCP treatment suggests a potential temporal shift in the functions of KCTD13 under conditions of stress. Since RAB5 is a GTPase involved in recruitment of RAB7, it is possible that the process of recruitment occurs at a faster speed, thus, the co-localization with RAB5 is not seen however strong co-localization occurs with RAB7. In comparison, the observed co-localization between GFP-KCTD13FL and RFP-RAB7 is retained and remains the same in the CCCP treated conditions (Figure 15B). The results from the co-localization with RAB5 and RAB7, with and without CCCP treatment, suggest a role of KCTD13 in the endosomal pathway. Reports have suggested a convergence between the endocytic pathways and autophagic vacuoles (Liou, Geuze, Geelen, & Slot, 1997). Thus, KCTD13 in association with Cul-3 and the endocytic vacuoles can potentiate the merge of endocytic structures with autophagic vacuoles. Table 2 summarizes the co-localization

of overexpressed KCTD13 constructs with endosomal markers: RAB5, RAB7, RAB-11A and RAB-11B (Table 2).

Expressed KCTD13 Localizes with Autophagy Proteins ATG12 and ATG13 and Autophagy Marker LC3

To determine whether KCTD13 may play a role in the convergence of the endocytic and autophagic pathways, co-localization of the protein with known autophagy markers and proteins were performed. P62 or Sequestosome-1 (SQSTM1), an autophagy receptor interacting directly with autophagy cargo, associates with GFP-KCTD13FL (Figure 17). Endogenous p62 is diffuse and does not co-localize with GFP-KCTD13FL without CCCP treatment (Figure 16). Treatment with CCCP, however, increases the number of endogenous p62 puncta and GFP-KCTD13FL puncta, which appear to concentrate in areas adjacent to each other (Figure 17). The association between endogenous p62 and GFP-KCTD13FL after CCCP treatment suggests a function of KCTD13 in autophagy distinct from p62. .

To determine whether KCTD13 is involved in autophagy, co-localization with autophagy markers ATG12, ATG13 and LC3 were performed. ATG12, a ubiquitin like protein involved in the formation of autophagy vesicles through conjugation with ATG5 (Yang & Klionsky, 2010b). HA-KCTD13FL puncta does not co-localize with cytoplasmic GFP-ATG12 without CCCP treatment (Figure 17A). However, CCCP treatment results to HA-KCTD13FL and GFP-ATG12 co-localization marked with distinct morphological changes in the structures of both proteins. HA-KCTD13FL and

GFP-ATG12 both change morphologies from punctate and cytoplasmic, respectively, to strand-like structures reminiscent of an expanding vesicular body (Figure 17A). The strand-like structures formed under CCCP conditions shows strong co-localization in the nucleus however, the co-localization is also seen in areas in the cytoplasm (Figure 18B, data not shown). Similar to GFP-ATG12, GFP-ATG13 also co-localizes with HA-KCTD13FL in CCCP treated cells (Figure 19A). ATG13 is an autophagy factor required for the formation of the autophagosome (Yang & Klionsky, 2010c). Without CCCP treatment, GFP-ATG13 is not visible in HeLa cells suggesting a possible high rate of GFP-ATG13 turnover or degradation (Figure 18). However treatment with CCCP reveals a co-localization of HA-KCTD13FL and GFP-ATG13 suggesting a possible role of KCTD13 in stabilizing the levels of ATG13 under stress conditions (Figure 18). HA-KCTD13FL and GFP-ATG13 co-localizes in the cytoplasm forming huge vesicle-like structures. However, the predominant co-localization of HA-KCTD13FL and GFP-ATG13 is nuclear with strand-like morphology noticeably excluding nucleoli (Figure 18B). The co-localization observed between KCTD13 and ATG12 and ATG13 after CCCP treatment suggests a potential role of KCTD13 in the early steps of autophagy.

To further elucidate a potential role of KCTD13 in autophagy, co-localization with canonical autophagy marker LC3 was performed. HA-KCTD13 constructs co-localize with GFP-LC3B (Figure 19). HA-KCTD13FL co-localizes with punctate GFP-LC3 near the nucleus (Figure 19A). Similar to the results with HA-KCTD13FL, HA-KCTD13BTB also co-localizes with GFP-LC3 forming extra-nuclear puncta, not normally seen with KCTD13BTB alone (Figure 19B). HA-KCTD13C also co-localizes with punctate GFP-LC3, however, the co-localization is reduced compared to expressed

KCTD13FL and BTB (Figure 19C). Treatment with CCCP however abolishes the observed co-localization of HA-KCTD13FL and GFP-LC3. GFP-LC3, similar to conditions without CCCP, is punctate however the amount of puncta is visually greater in CCCP treated cells (Figure 19D). The increased GFP-LC3 puncta however, does result in an increased co-localization between GFP-LC3 and HA-KCTD13 in CCCP treated cells thus, abolishing the co-localization observed in cells without CCCP treatment (Figure 20D). The disappearance of co-localization between HA-KCTD13 and GFP-LC3 with CCCP treatment, however, is a potential result of an up-regulation of autophagy in cells treated with CCCP. Thus, resulting to a faster turnover and disappearance of the observed binding between HA-KCTD13FL and GFP-LC3 seen in non-CCCP treated cells.

The co-localization of HA-KCTD13 and GFP-LC3 is consistent with ATG12 and ATG13, further implicating a role of KCTD13 in autophagy. The result also implies a potential role of KCTD13 in basal autophagy as seen from the co-localization with LC3 without treatment. Taken together, these results suggest a potential role of KCTD13 in mitochondrial homeostasis through the autophagy pathway via its interaction with Cul-3.Table 3 summarizes the co-localization of overexpressed KCTD13 with autophagy proteins: p62, ATG12, ATG12 and LC3B (Table 3).

Endogenous KCTD13 Expression

To rule out any possible overexpression artifacts and to determine the endogenous expression of KCTD13 in mammalian tissues, a KCTD13 antibody (Cell Signaling) was used to detect the protein. Using KCTD13 antibody, a nuclear localization with granular

structures was observed (Figure 20A and 20B). However, we cannot rule out nonspecific antibody binding. To further address this point in the future, HeLa cells will be transfected with Cul-3 to determine whether the structures observed will undergo morphological changes as seen with the overexpressed KCTD13 constructs. This future experiment will be a strong evidence for the endogenous expression of KCTD13 and will further support the interaction between the proteins in Cul-3 seen through immunofluorescence. Western blot analysis of a female wild type 129 mouse detects one major band in various tissues (Figure 20). Mouse tissue samples were normalized at 0.67 μg/μL using a BCA assay before loading. KCTD13, predicted at 36.4 kDa, is present in the heart (44.7 kDa), lung (43.6 kDa), spleen (35.6 kDa), ovary (40.3 kDa) and brain (62.7 kDa). The detection of these different size bands in different tissues suggests possible expression of KCTD13 splice variants in different tissues, though we cannot rule out non-specific antibody interactions. Alternatively, varying sizes of the bands detected could also suggest posttranslational modifications in different tissues possibly through ubiquitination, proteolytic cleavage or other. (Figure 20C)

DISCUSSION

Since the discovery of KCTD13, no other study has attempted to characterize KCTD13. This study presents the most recent attempt to characterize the protein and unravel other molecular functions of KCTD13 previously not known or considered. The results show co-localization of expressed KCTD13 with expressed Cul-3 and mitochondria after CCCP treatment suggesting a potential role of KCTD13 mitochondrial homeostasis. In support of this observation, expressed KCTD13 co-localizes with endosome markers, RAB5 and RAB7 and autophagy marker LC3 along with autophagy proteins Atg12 and Atg13. These results, taken together, suggest a previously unreported potential role of KCTD13 mitochondrial homeostasis via autophagy.

The molecular pathway of autophagy in mammals has only been beginning to be uncovered as understanding of the pathway derives from yeast genetic studies (Huang & Klionsky, 2002). Mammalian homologs of the yeast ATG genes have been uncovered recently although many genes discovered in yeast still do not have a determined mammalian homolog. KCTD proteins, being yeast Whi2 homologs, are thus great candidates for the elucidation of the molecular process of autophagy in mammalian cells (Chen et al, 2013). Expression of KCTD13 revealed mitochondrial localization, a finding previously not reported. As BTB domain containing proteins, KCTD13 and other members of the family have been predicted to bind Cul-3 (Skoblov et al., 2013). Binding of KCTD13 and Cul-3 has not been observed experimentally although the results of this study seem to support the hypothesis through the co-localization observed between expressed KCTD13 and Cul-3. However, more in depth experiments need to be performed to determine protein-protein interaction. The co-localization of expressed

KCTD13 with Cul-3 implied a potential role of the protein in the CRL E3 ubitquitin-protein ligase complex which mediates the ubiqtuination and degradation of target proteins (Krek, 2003). Indeed, CCCP treatment incudes a strong co-localization between KCTD13, Cul-3 and the Mitochondria suggesting a role of KCTD13 in the process of protein ubiqutination and degradation through the Cul3-RING-BCR E3 complex. In support of this finding, PINK1 (PTEN-induced putative kinase 1) has been shown to recruit Parkin, an E3 ubiquitin ligase ubiquitylating damaged mitochondria, from the cytoplasm to the mitochondria initiating degradation through autophagy (Matsuda et al., 2010). Thus, KCTD13, like PINK1, potentially plays a role in the degradation of damaged mitochondria through the autophagy.

The potential role of KCTD13 in autophagy is a novel finding. No reports have been made regarding the involvement of KCTD13 in the pathway although other members of the KCTD family have been implicated (Metz & Hardwick, Unpublished) (Ladha, 2014). The exact role of KCTD13 in the pathway however, remains to be determined. Co-localization studies performed with expressed KCTD13 suggests its potential role in the delivery of ubiquitinated mitochondria to either the endosomes or in the phagophore (Figure 21). Indeed, KCTD13 co-localizes with endosome markers RAB5 and RAB7 suggesting a possible role of the protein in delivery of cargo in the endosomal pathway. However, the observed co-localization can also denote the degradation of expressed KCTD13 through the endocytic pathway. Thus, further work needs to be done to elucidate the observed co-localization of KCTD13 with endosomal markers. Perhaps detection of endogenous endosomal markers with KCTD13 will provide a better understanding of the co-localization observed in this study. KCTD13, in

addition to endosomal markers also, co-localize with autophagy marker LC3 and autophagy proteins ATG12 and ATG13. ATG12, in conjugation with Atg5, and LC3 are involved in the nucleation events of the phagosome (Yorimitsu & Klionsky, 2005) thus suggesting a role of KCTD13 in the delivery of cargo in the growing phagophore. The findings, however, are not conclusive to directly identify KCTD13 in the selective degradation of damaged mitochondria. Further studies need to confirm whether the co-localization observed between KCTD13 and the markers tested in this study are observable under different methods (i.e. biochemical) and conditions such as starvation, a potent activator of autophagy. The findings of this study, however, provide a new perspective on the understanding of the etiology and pathogenesis of neuropsychiatric disorders such as ASD.

The implication of KCTD13 in the development of ASD was groundbreaking (Golzio et al., 2012) however molecular mechanisms involving the gene in the observed ASD phenotypes have not well been elucidated. The potential role of KCTD13 in autophagy presents a new perspective in understanding the molecular role of KCTD13 in ASD. Consistent with findings of autophagy defects in neurodegenerative disorders, a potential defect in autophagy mediated by KCTD13 possibly leads to the development of ASD. However, more research needs to be done in order to completely verify and specify the role of KCTD13 in autophagy and ASD. The research presented in this study, hopefully, will shed some light on the new novel role of KCTD13 in autophagy and stimulate research in understanding the connection between KCTD13 and ASD.

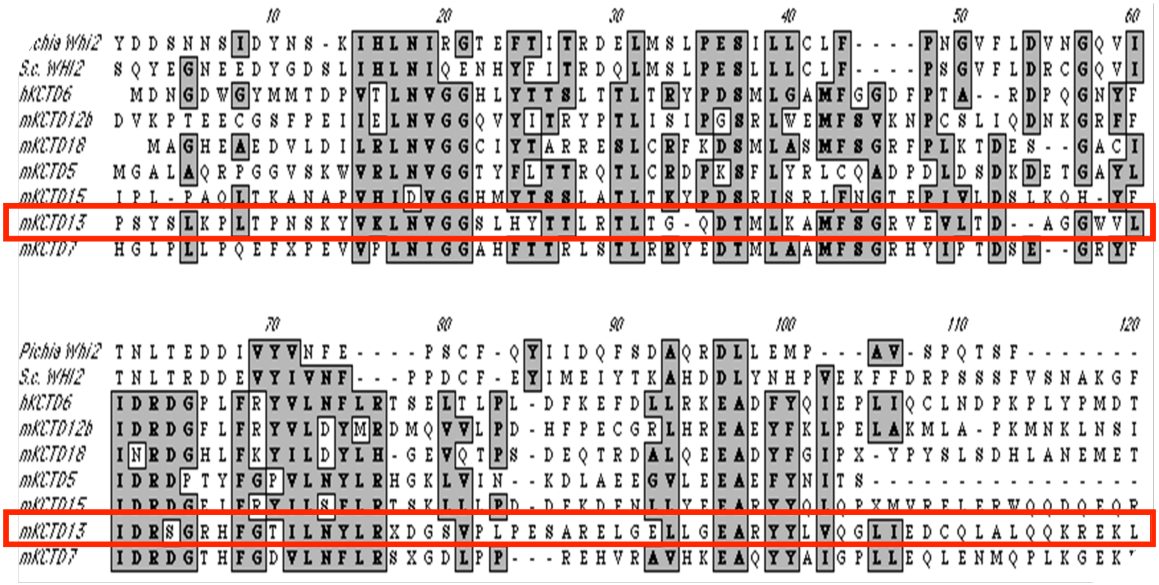

Figure 1. *KCTD13* is a mammalian yeast *Whi2* homolog. Yeast Whi2 is an essential gene for nutrient sensing and survival. Sequence alignment shows the yeast gene has mammalian homologs. Above is the sequence for the Whi2 gene in *Saccharomyces cervisiae* and below are different mammalian KCTD proteins. KCTD13 is second to the last sequence. Source: (Teng et al., 2013).

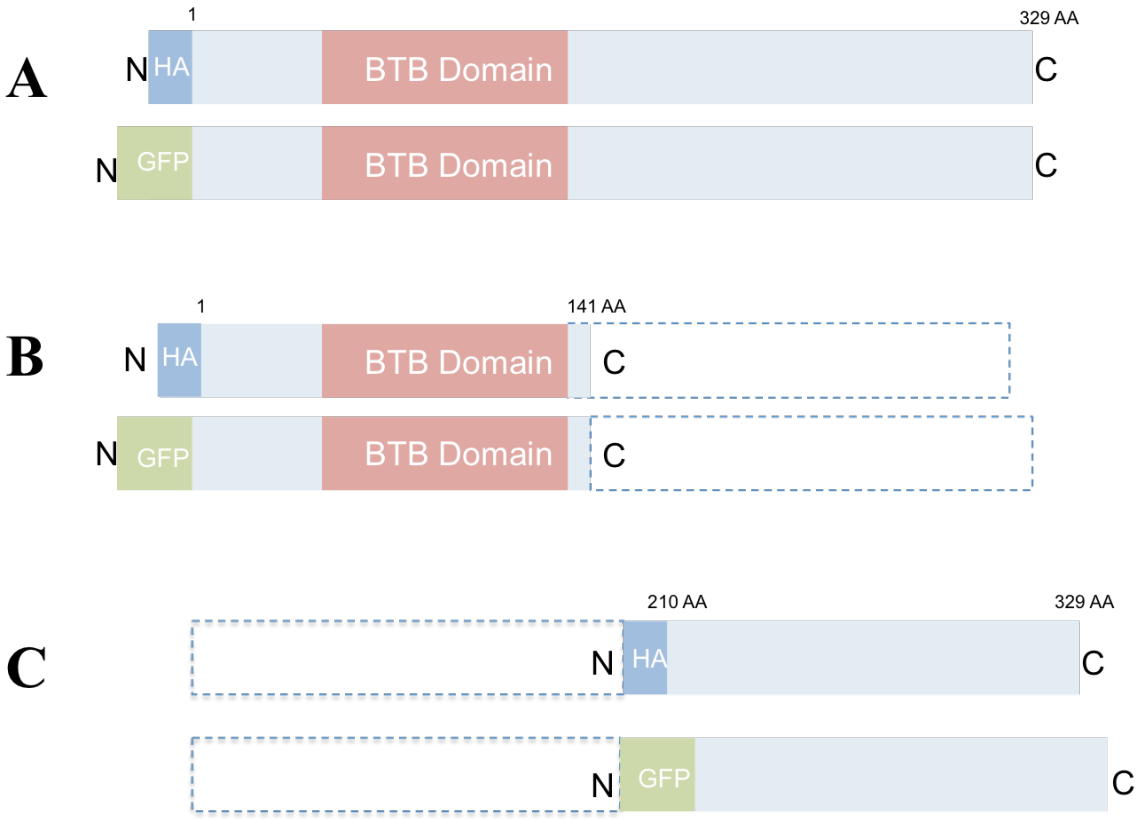

Figure 2. Schematic of KCTD13 Constructs. **(A)** Schematic of Full Length human KCTD13 protein. KCTD13 is a 329 amino acid protein with a conserved BTB domain in the N-terminus similar to other members of the KCTD family. The full-length protein was tagged with hemaglutanin (HA) or green fluorescence protein (GFP) on the N-terminus for detection using immunofluorescence microscopy. **(B)** Schematic of human KCTD13 BTB construct (aa 1-141). The N-terminus was tagged with HA or GFP. **(C)** Schematic of human KCTD13 C-terminus construct. The construct was created from the full length KCTD13 protein by truncation of the first 141 amino acids and fused at its N-terminus to HA or GFP.

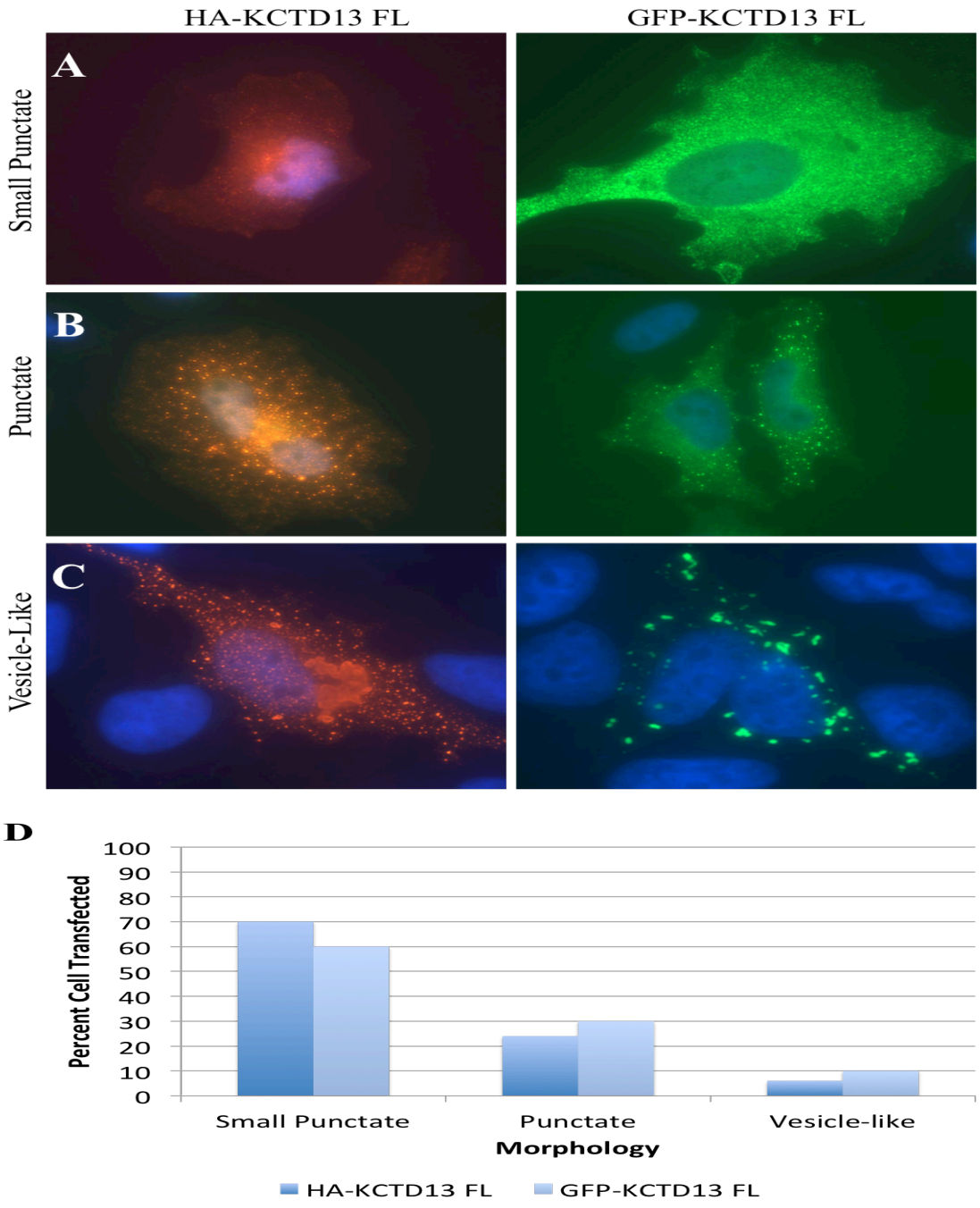

Figure 3. Expression of KCTD13 FL in HeLa cells Leads to Formation of Cytoplasmic Structures. (A) Overexpressed KCTD13 FL, either HA or GFP tags form cytoplasmic structures with three distinct morphologies in HeLa cells. The most common is the diffuse cytoplasmic morphology. **(B)** Example of punctate morphology, **(C)** Examples of bleb-like morphology. (D) Quantification of cell morphologies by percent of transfected cells, counting 200-300 cells per condition in a single experiment

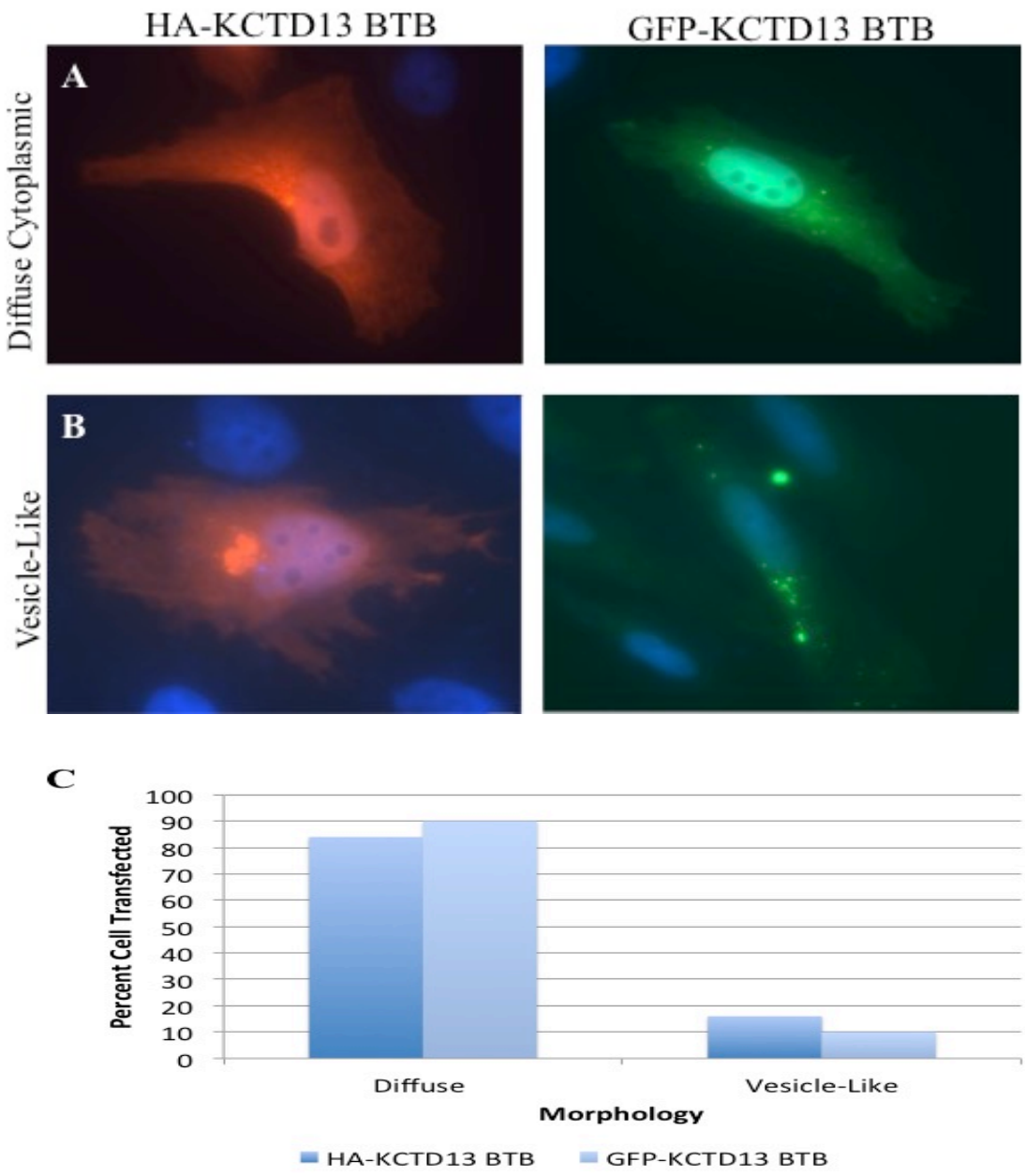

Figure 4. Expression of KCTD13 BTB in HeLa Cells Leads to Formation of Diffuse-Cytoplasmic and Vesicle-like Structures. **(A)** Overexpression of KCTD13 BTB leads to the formation of two cytoplasmic morphologies, the most common being diffuse cytoplasmic morphology (~90% of transfected cells). **(B)** Overexpression of KCTD13 BTB also leads to a vesicle-like morphology. **(C)** Quantification of cell morphologies by percent cell transfected, counting 200-300 cells per condition in a single experiment.

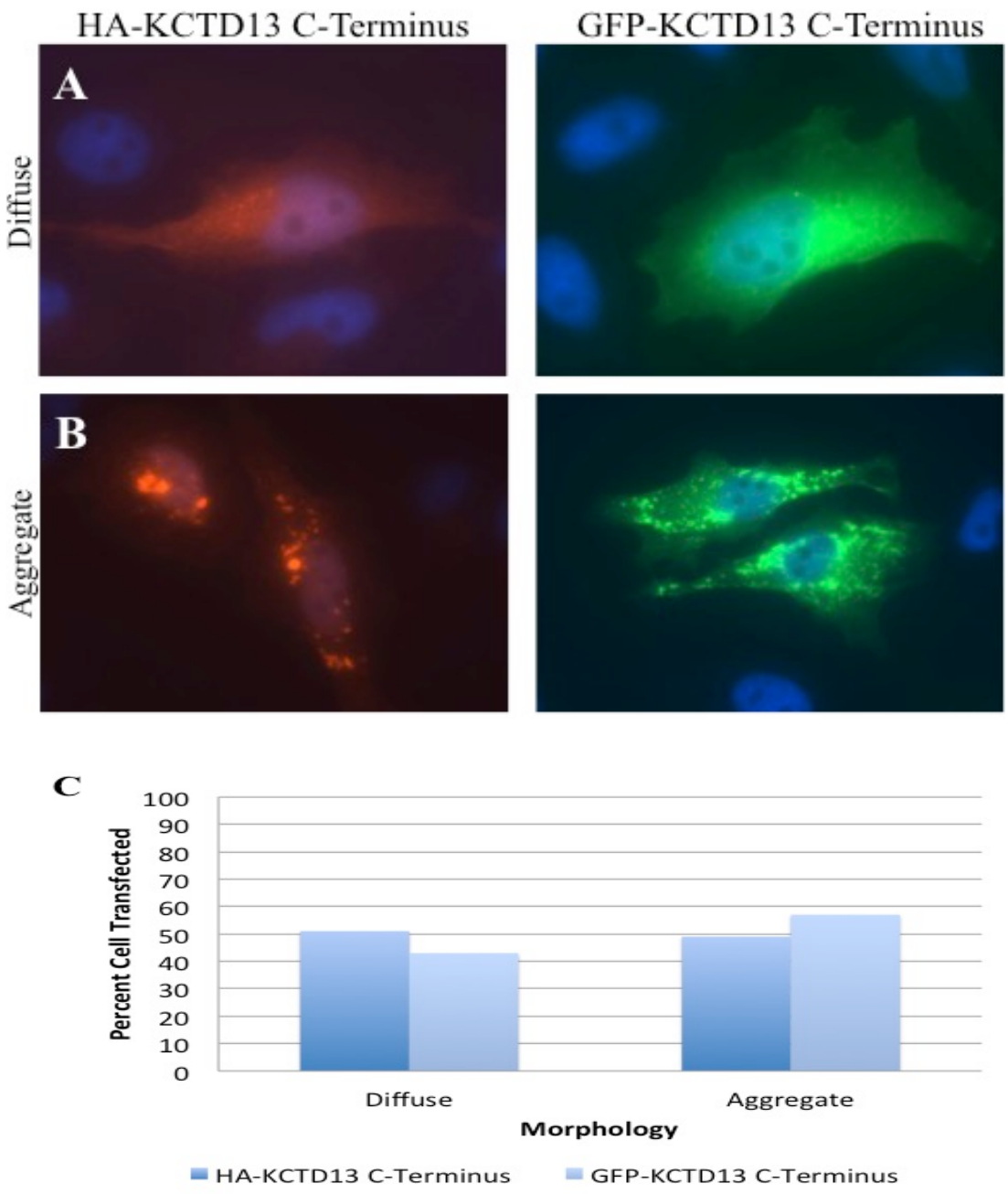

Figure 5. Expression of KCTD13 C-terminal constructs in HeLa Cells Leads to the Formation of Diffuse and Aggregate Like Structures in the Cytoplasm. About half of the cells expressing KCTD13C form diffuse cytoplasmic structures in HeLa cells **(A)**, while the other half forms aggregate-like structures, which are located near or around the nucleus **(B)**. **(C)** Quantification of cell morphology by percent of transfected cells counting 200-300 cells per condition in a single experiment.

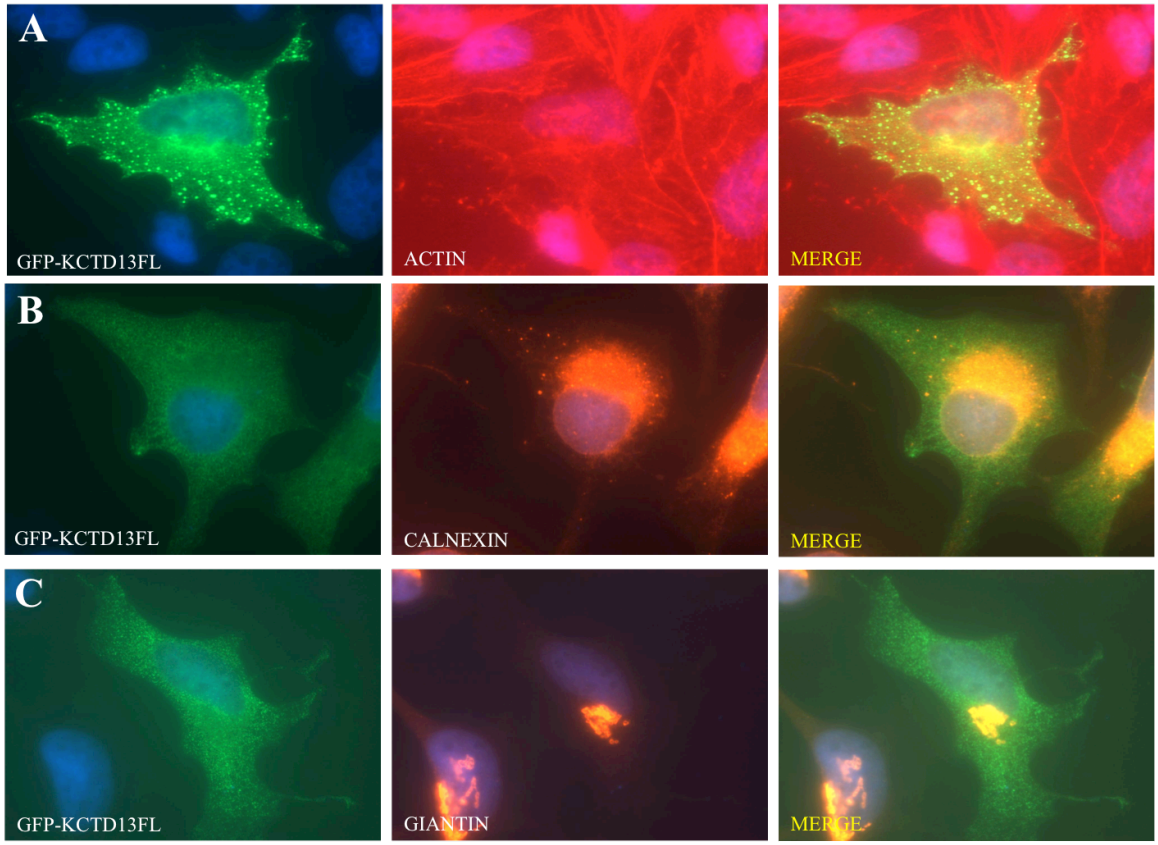

Figure 6. KCTD13FL does not co-localize with the Cytoskeleton, endoplasmic reticulum and Golgi. (A) GFP-KCTD13FL does not co-localize with F-actin stained with Phalloidin. However, the puncta formed by KCTD13FL seems to associate with the stained actin. **(B)** GFP-KCTD13FL does not co-localize with calnexin, a marker for the endoplasmic reticulum, **(C)** nor with Giantin, a marker for the Golgi.

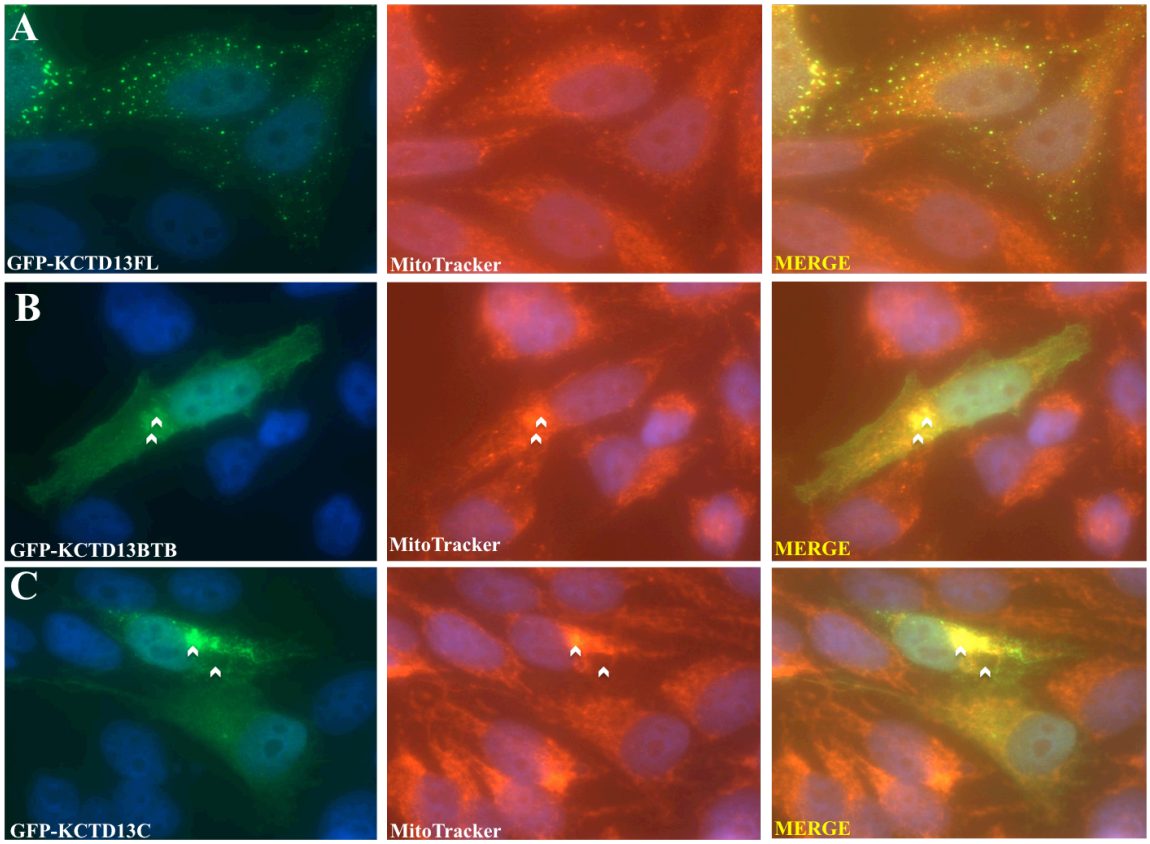

Figure 7. KCTD13BTB and GFP-KCTD13C Co-localize with Mitochondria while KCTD13FL associates with Mitochondria. (A) GFP-KCTD13FL associates with the mitochondrial dye MitoTracker. The punctate morphology of KCTD13FL is almost always found near MitoTracker-labeled mitochondria suggesting an association. **(B)** GFP-KCTD13BTB co-localizes with MitoTracker in most cells. **(C)** GFP-KCTD13C co-localizes with Mitotracker. The aggregate morphology but not the diffuse morphology of KCTD13 C-terminus co-localizes with MitoTracker.

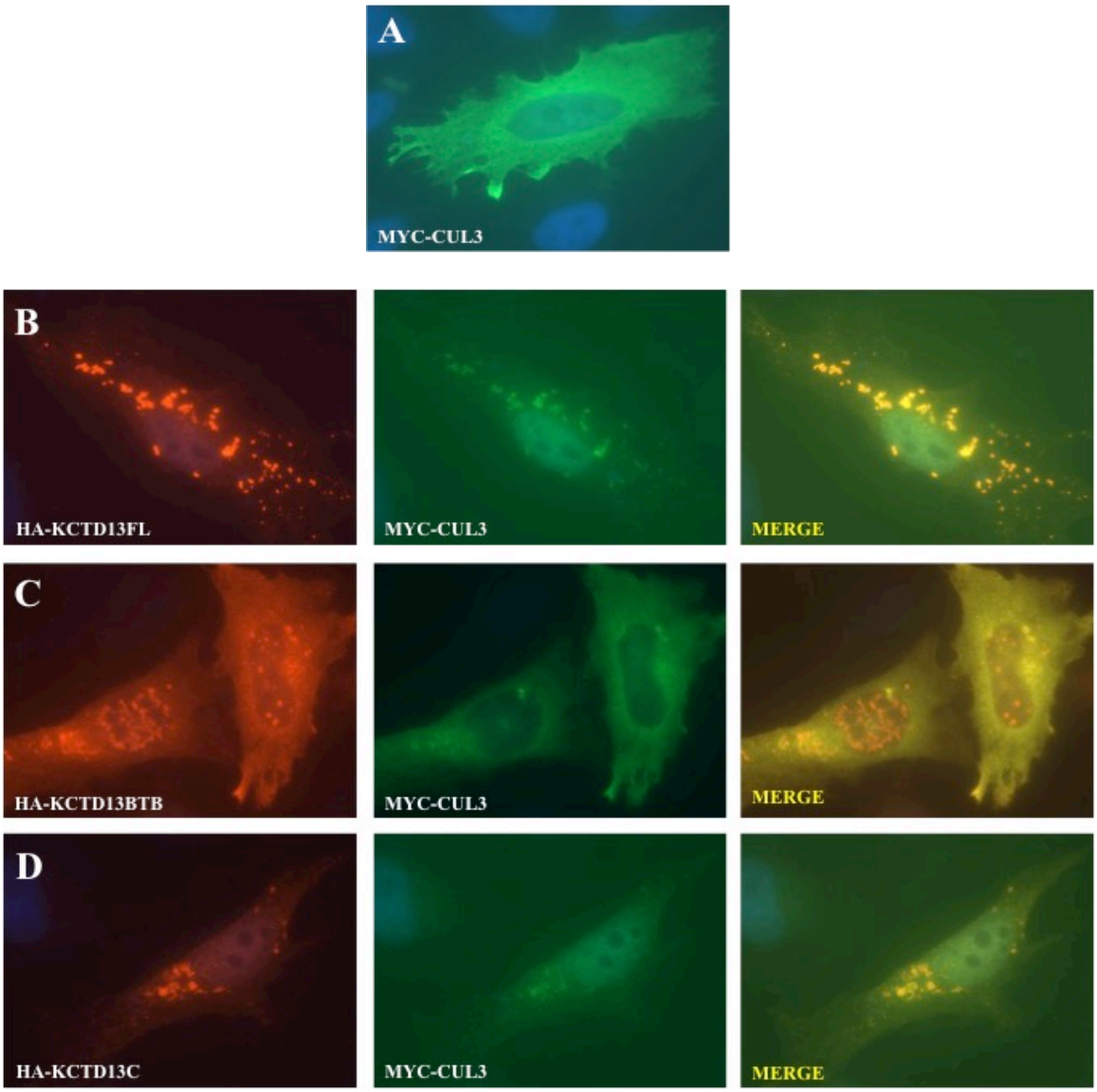

Figure 8. KCTD13 Constructs Co-localize with Cullin-3. (A) Myc- Cullin-3 is diffuse in HeLa cells without transfected KCTD13. **(B)** Co-expression of HA-KCTD13 FL with Myc-Cul-3 results to the co-localization of the proteins in vesicle-like structures. **(C)** Co-expression of HA-KCTD13BTB and Myc-Cul-3 leads to the cytoplasmic localization of HA-KCTD13BTB and Myc-Cul-3 and an accumulation of KCTD13BTB structures in the nucleus. **(D)** Co-expression of HA-KCTD13C and Myc-Cul-3 results in co-localization in the cytoplasm in distinct aggregate-like structures.

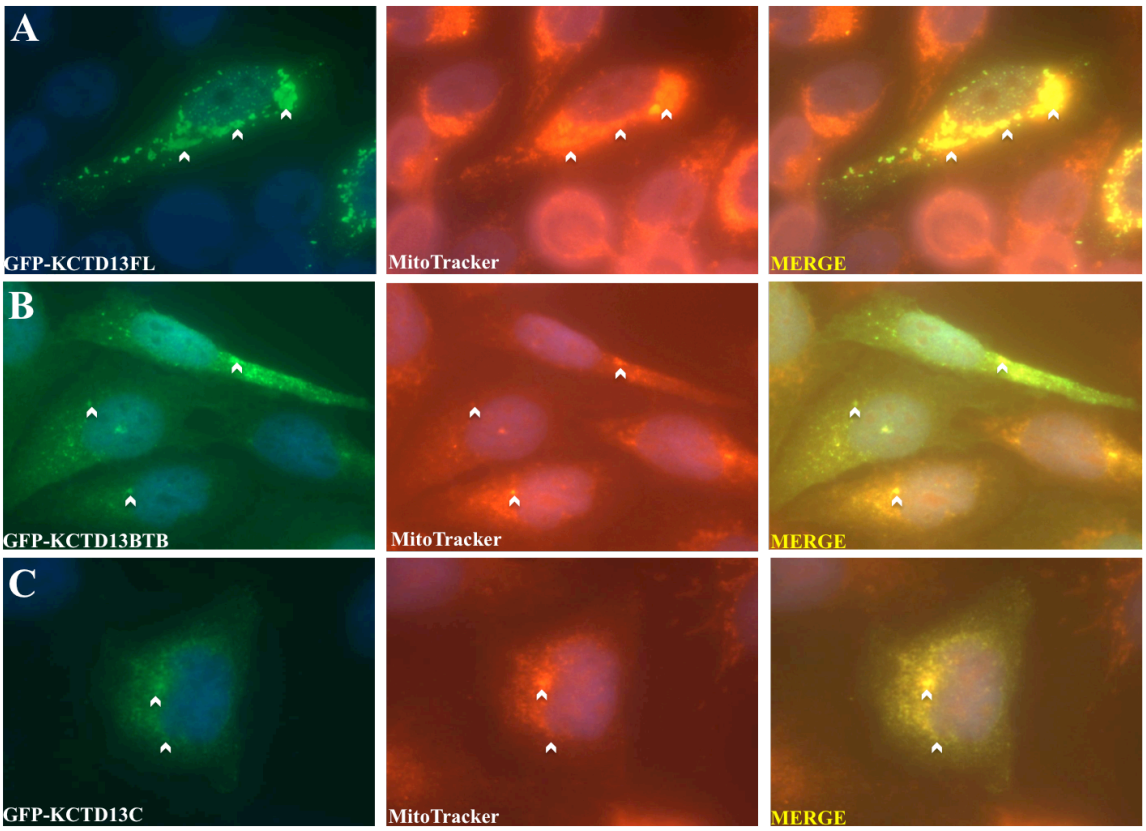

Figure 9. KCTD13 Constructs Strongly co-localize with Mitochondrial Marker MitoTracker after CCCP treatment. (A) GFP-KCTD13 FL co-localizes with MitoTracker after treatment CCCP in vesicle like structures. **(B)** GFP-KCTD13 BTB also co-localizes with MitoTracker after treatment with CCCP. **(C)** Like GFP-KCTD13FL and BTB, GFP-KCTD13C also co-localizes with MitoTracker after CCCP treatment.

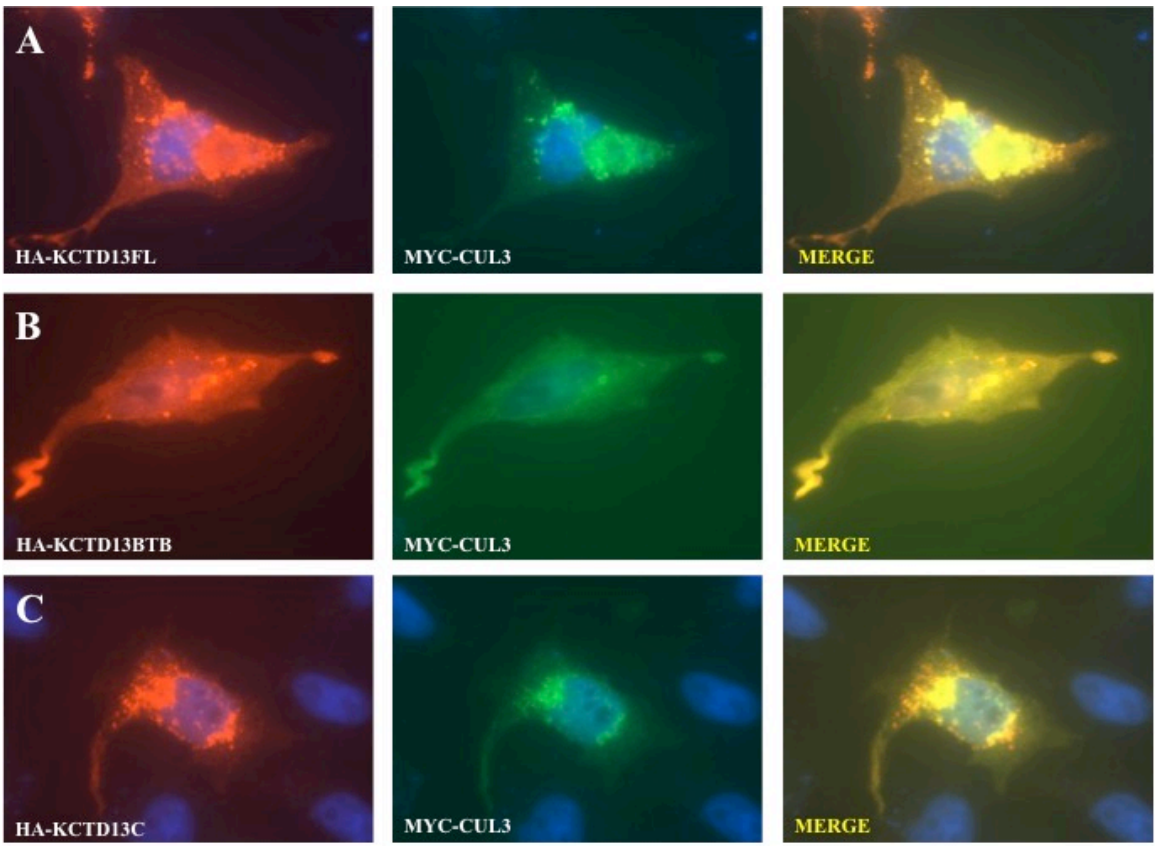

Figure 10. KCTD13 Constructs localize with Cul-3 after CCCP treatment. (A) Expressed KCTD13FL co-localizes with Myc-Cul3 in vesicle-like structures. **(B)** HA-KCTD13BTB also co-localizes with Myc-Cul-3 in the cytoplasm and results to the disappearance of the KCTD13BTB structures inside the nucleus seen without CCCP treatment. **(C)** HA-KCTD13C co-localizes with Myc-Cul-3 in aggregate like structures.

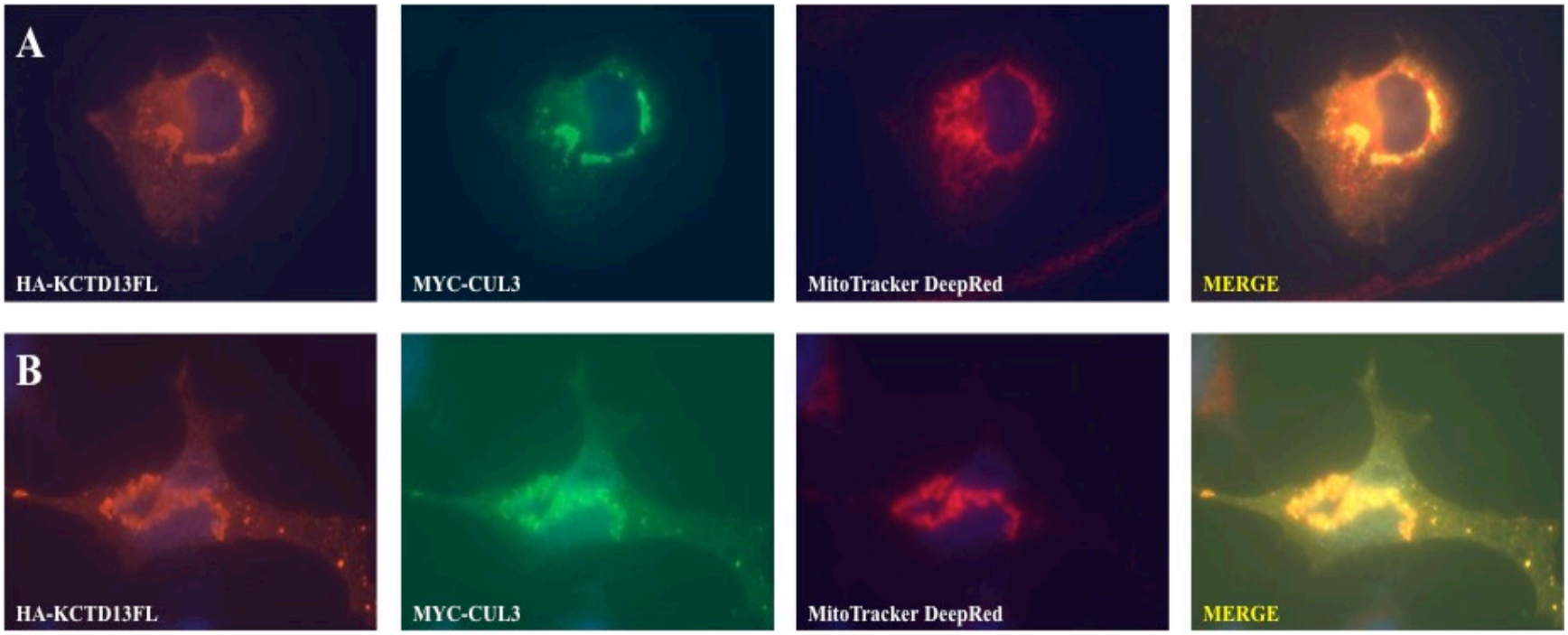

Figure 11. KCTD13FL, Cullin-3 and Mitochondria Co-localize Strongly after CCCP Treatment. (A) Without CCCP treatment, HA-KCTD13FL and Myc-Cul-3 co-localize in areas surrounding the nucleus. **(B)** Treatment with CCCP results in the co-localization of HA-KCTD13FL, Myc-Cul-3 and mitochondria.

Sub-Cellular Organelle (Marker)	Without Treatment	With CCCP Treatment (25 uM for 4 hours)
Actin (Phalloidin-Stain)	No Co-localization	No Co-localization
Endoplasmic Reticulum (Calnexin)	No Co-localization	No Co-localization
Golgi (Golgi)	No Co-localization	No Co-localization
Mitochondria (MitoTracker)	FL: Association	FL: Co-localization
	BTB: Co-localization	BTB: Co-localization
	C-Terminus: Co-localization	C-Terminus: Co-localization

Abbreviations: *FL: KCTD13 Full Length*
BTB: KCTD13 BTB
C: KCTD13 C-terminus

Table 1. Summary of KCTD13 Co-localization with Subcellular Organelles. Expressed KCTD13FL, BTB and C co-localize with MitoTracker with and without CCCP treatment.

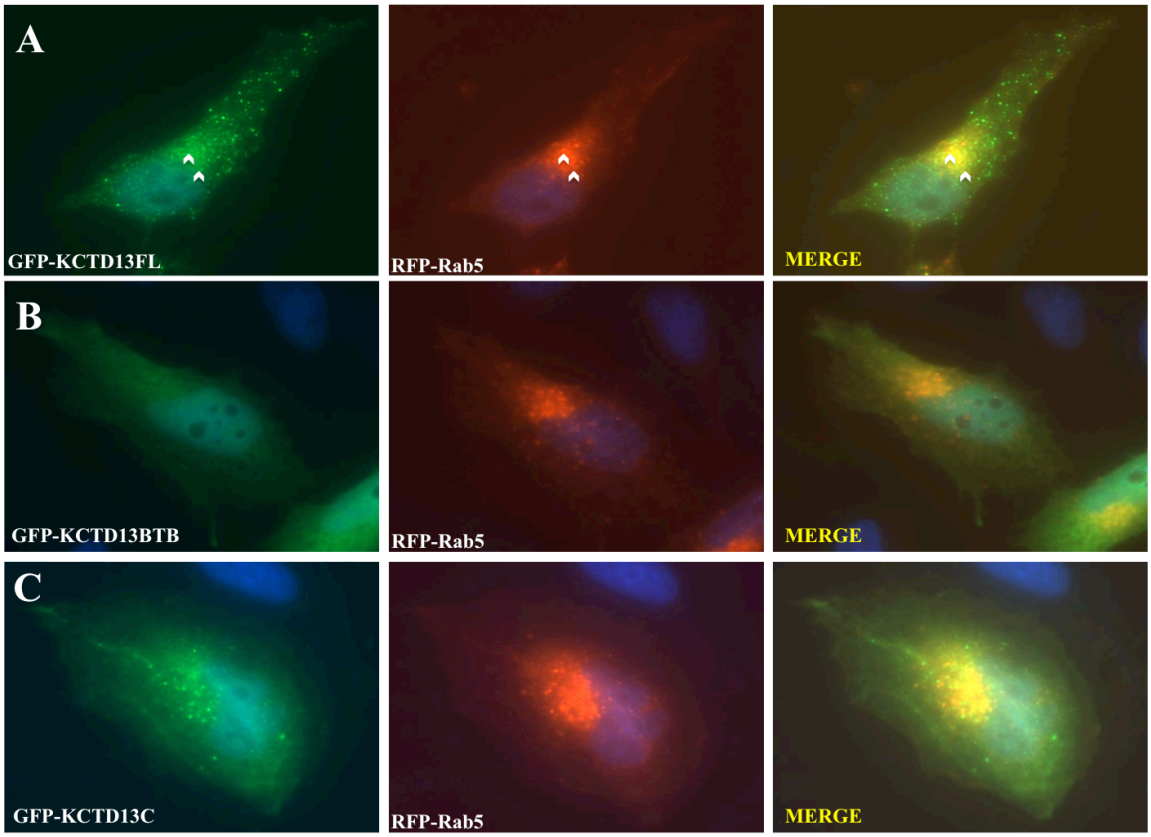

Figure 12. KCTD13FL Co-localizes with Endosome Marker RAB5 but not KCTD13 BTB and C. (A) GFP-KCTD13FL co-localizes with overexpressed RAB5 in punctate like structures. **(B)** GFP-KCTD13BTB is diffuse and does not co-localize with RAB5 puncta. **(C)** GFP-KCTD13C, like KCTD13BTB, does not co-localize with punctate RAB5.

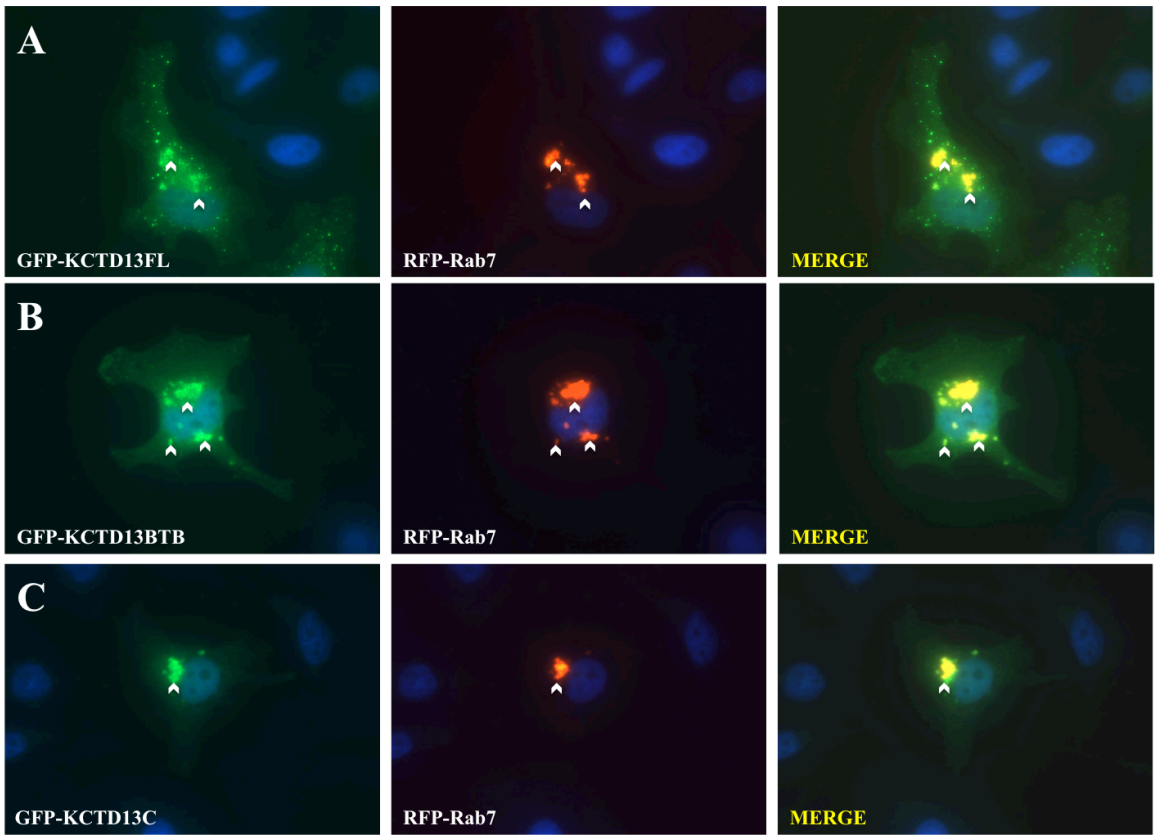

Figure 13. KCTD13FL, BTB and C Strongly Co-Localize with Endosome Marker RAB7. (A) GFP-KCTD13FL co-localizes with RAB7 in vesicle-like structures.**(B)** GFP-KCTD13BTB also co-localizes with overexpressed RAB7, forming vesicle-like structures near the nucleus. **(C)** Similar GFP-KCTD13FL and BTB, the GFP-KCTD13C also co-localizes with the vesicle-like RAB7.

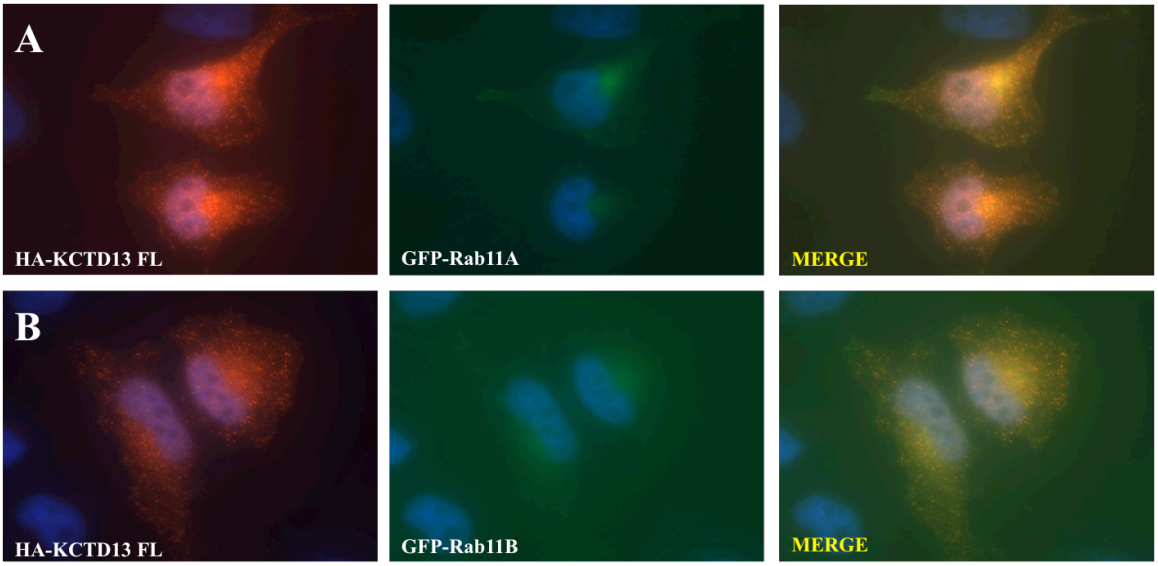

Figure 14. KCTD13FL Does Not Co-localize with Late Endosome Markers Rab11A and RAB11B. (A) HA-KCTD13 FL appears punctate but does not co-localize with GFP-RAB11A. **(B)** Similar to GFP-RAB11A, HA-KCTD13 FL also does not co-localize with GFP-RAB-11B.

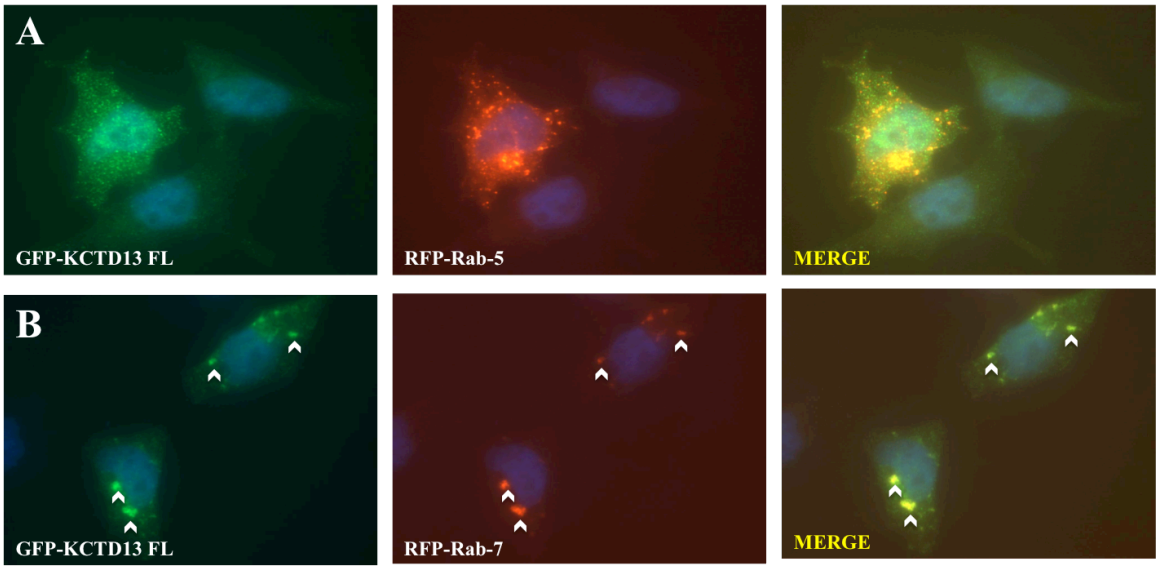

Figure 15. KCTD13FL Co-Localizes with Endosome Marker RAB7 After Treatment with CCCP but not with RAB5. (A) Treatment with the mitochondrial uncoupling agent CCCP changes the co-localization of GFP-KCTD13FL with RFP-RAB5. GFP-KCTD13FL does not co-localize with RFP-RAB5 after treatment with CCCP. **(B)** In contrast, CCCP treatment does not affect the co-localization of GFP-KCTD13FL and RFP-RAB7.

Endosomes	Without Treatment	With CCCP Treatment (25 uM for 4 hours)
Rab-5	FL: Co-localization	FL: Association
	BTB: No Co-localization	BTB: No Data
	C: No Co-localization	C: No Data
Rab-7	FL: Co-localization	FL: Co-localization
	BTB: Co-localization	BTB: No Data
	C: Co-localization	C: No Data
Rab-11A	FL: No Co-localization	FL: No Co-localization
Rab-11B	FL: No Co-localization	FL: No Co-localization

Abbreviations: *FL: KCTD13 Full Length*
BTB: KCTD13 BTB
C: KCTD13 C-terminus

Table 2: Summary of KCTD13 Co-localization with Endosome Markers. Expressed KCTD13 FL co-localizes with expressed RAB5 and RAB7, but not with RAB11-A and B. Expressed KCTD13 BTB and C co-localizes with expressed RAB7 but not RAB5. With CCCP treatment, the co-localization of expressed KCTD13 and expressed RAB7 is retained but not with expressed RAB5.

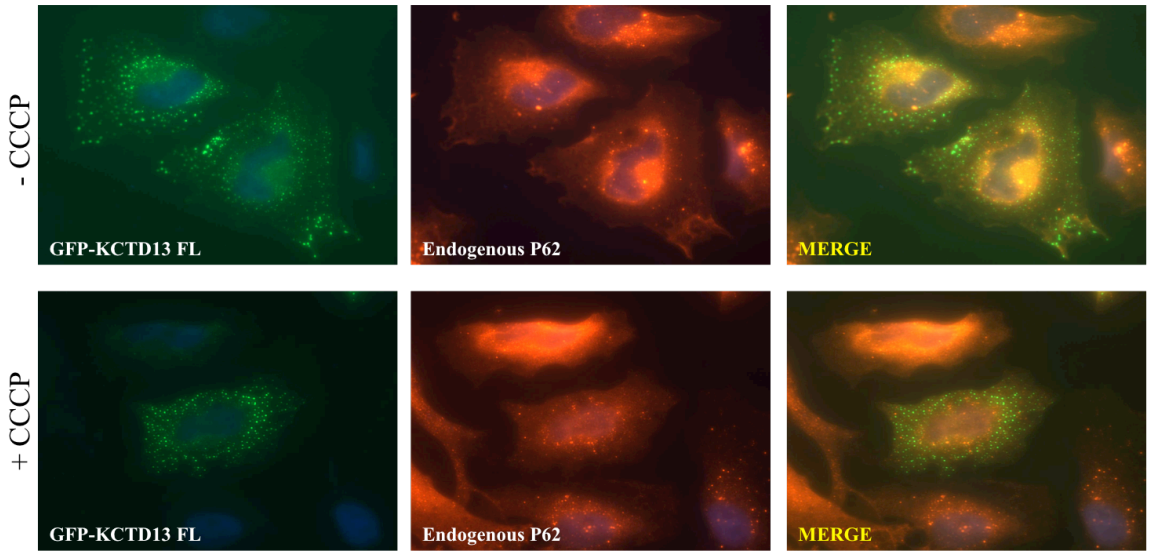

Figure 16. KCTD13FL Associates with p62 after CCCP Treatment. Endogenous p62 forms punctate structures. Expressed KCTD13FL does not seem to co-localize with endogenous p62. Treatment with CCCP, however, intensifies the puncta formation in p62 and expressed KCTD13FL resulting to an association between the two proteins.

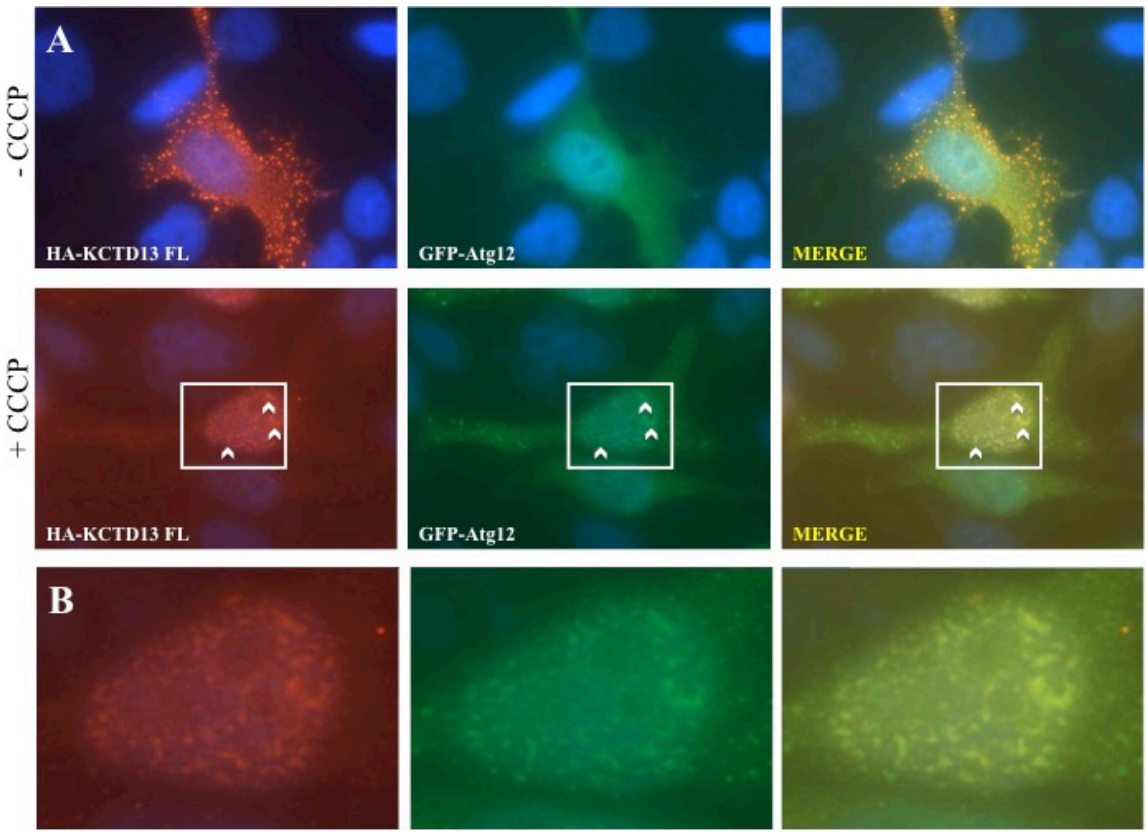

Figure 17. KCTD13FL co-localizes with Autophagy Related Protein ATG12 after CCCP treatment. (A) GFP-ATG12 forms diffuse cytoplasmic structures and does not co-localize with HA-KCTD13FL without CCCP treatment. However, treatment with CCCP changes the morphology of GFP-ATG12 and HA-KCTD13FL resulting in the formation of strand-like structures in the nucleus **(B)**.

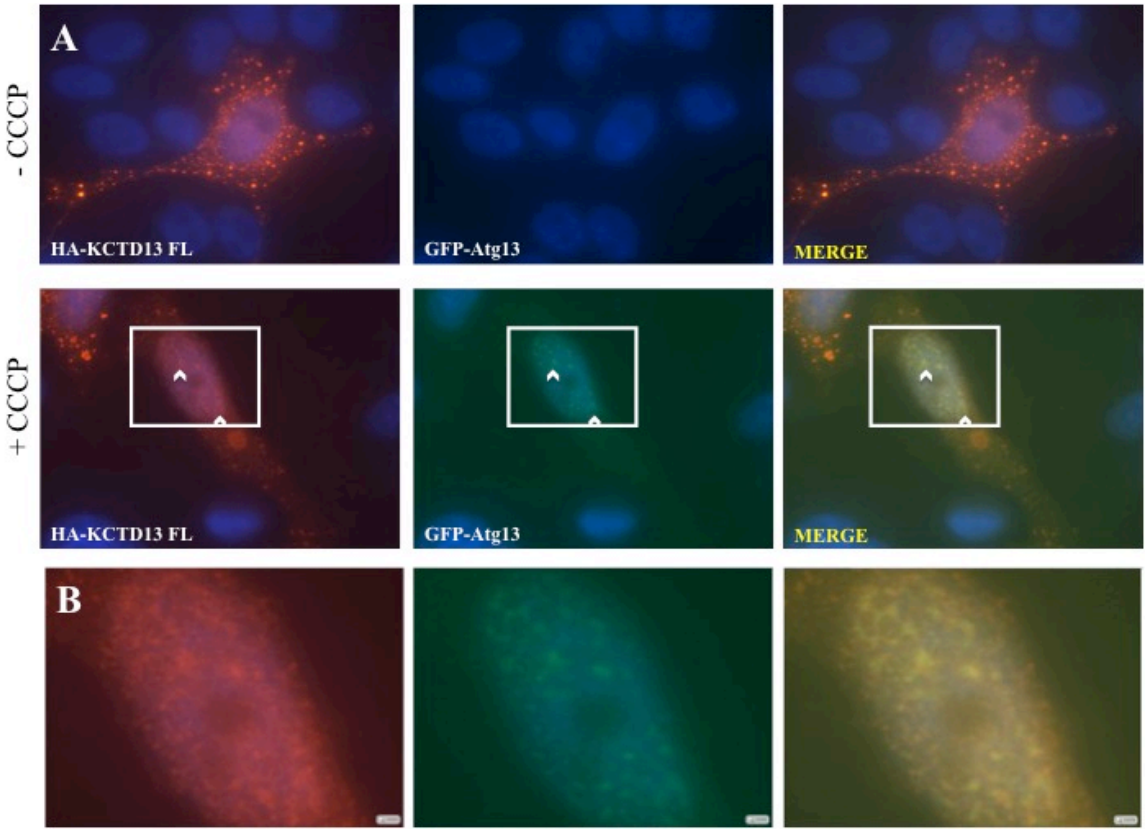

Figure 18. KCTD13FL co-localizes with Autophagy Related Protein ATG13 after CCCP treatment. (A) GFP-ATG13 is not visible in cells without treatment suggesting fast protein turnover or degradation. **(B)** Treatment with CCCP, however, reveals a strand-like morphology of GFP-ATG13 in the nucleus where HA-KCTD13FL co-localizes.

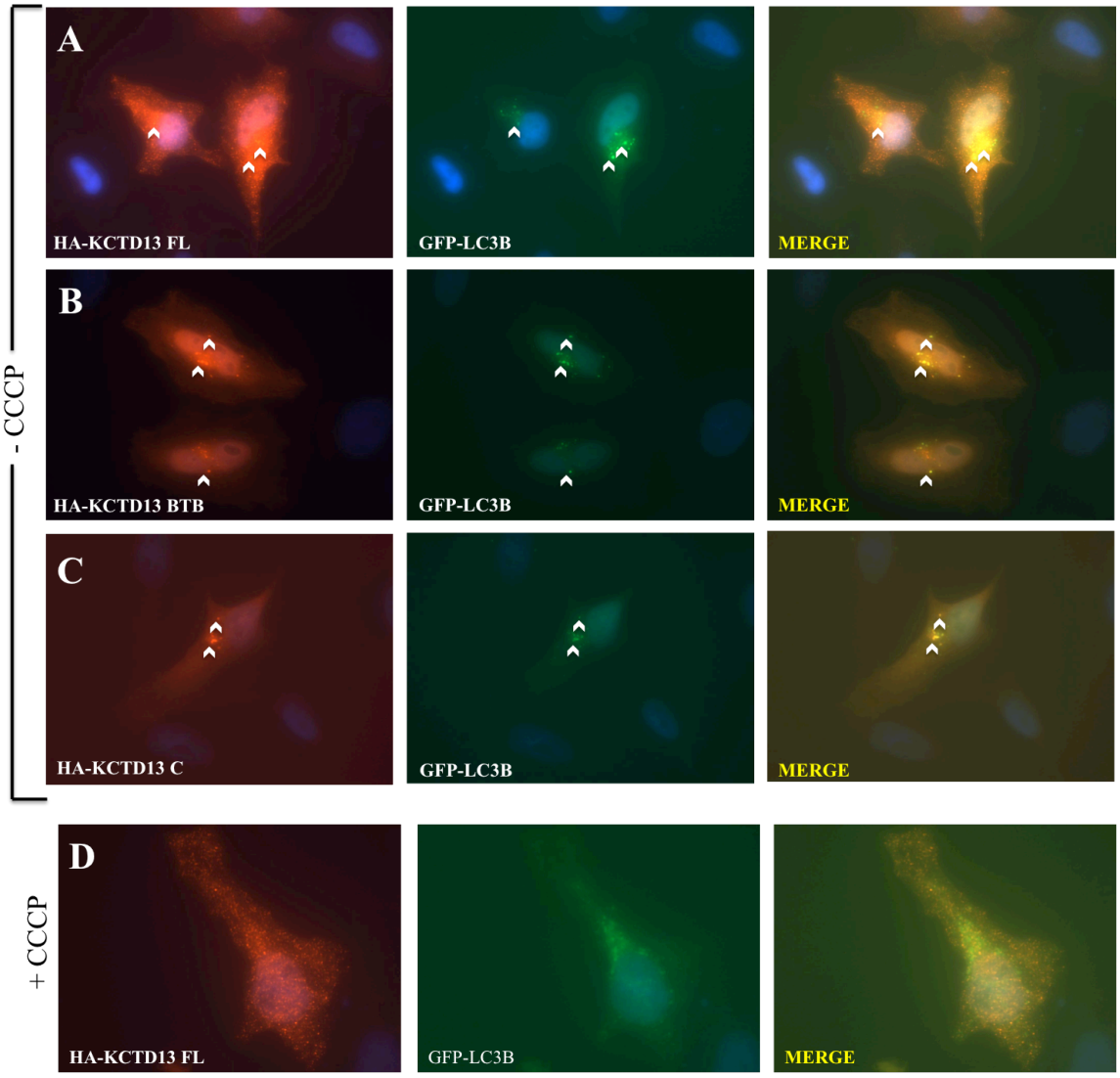

Figure 19. KCTD13FL, BTB and C Co-localize with Expressed Autophagy Marker LC3B. (A) GFP-LC3B co-localizes with the HA-KCTD13 FL. **(B)** HA-KCTD13BTB also co-localizes with the GFP-LC3B puncta in distinct areas near the nucleus. **(C)** Similar to the co-localization of HA-KCTD13BTB, HA-KCTD13C also co-localizes with GFP-LC3B. **(D)** Treatment with CCCP, however, abolishes the co-localization of HA-KCTD13FL and LC3B.

Autophagy Markers	Without Treatment	With CCCP Treatment (25 uM for 4 hours)
P62	No Co-localization	Association
ATG12	No Co-localization	Co-localization
ATG13	No Co-localization	Co-localization
LC3B	FL: Co-localization	FL: No Co-localization
	BTB: Co-localization	BTB: No Data
	C: Co-localization	C: No Data

Abbreviations: *FL: KCTD13 Full Length*
BTB: KCTD13 BTB
C: KCTD13 C-terminus

Table 3: KCTD13 Co-localization with Autophagy Markers. P62 associates with expressed KCTD13FL with CCCP treatment while ATG12 and ATG13 co-localize with expressed KCTD13FL after treatment with CCCP. Expressed KCTD13FL, BTB and C co-localize with canonical autophagy marker LC3B without CCCP treatment however, treatment with CCCP abolishes co-localization with the KCTD13FL.

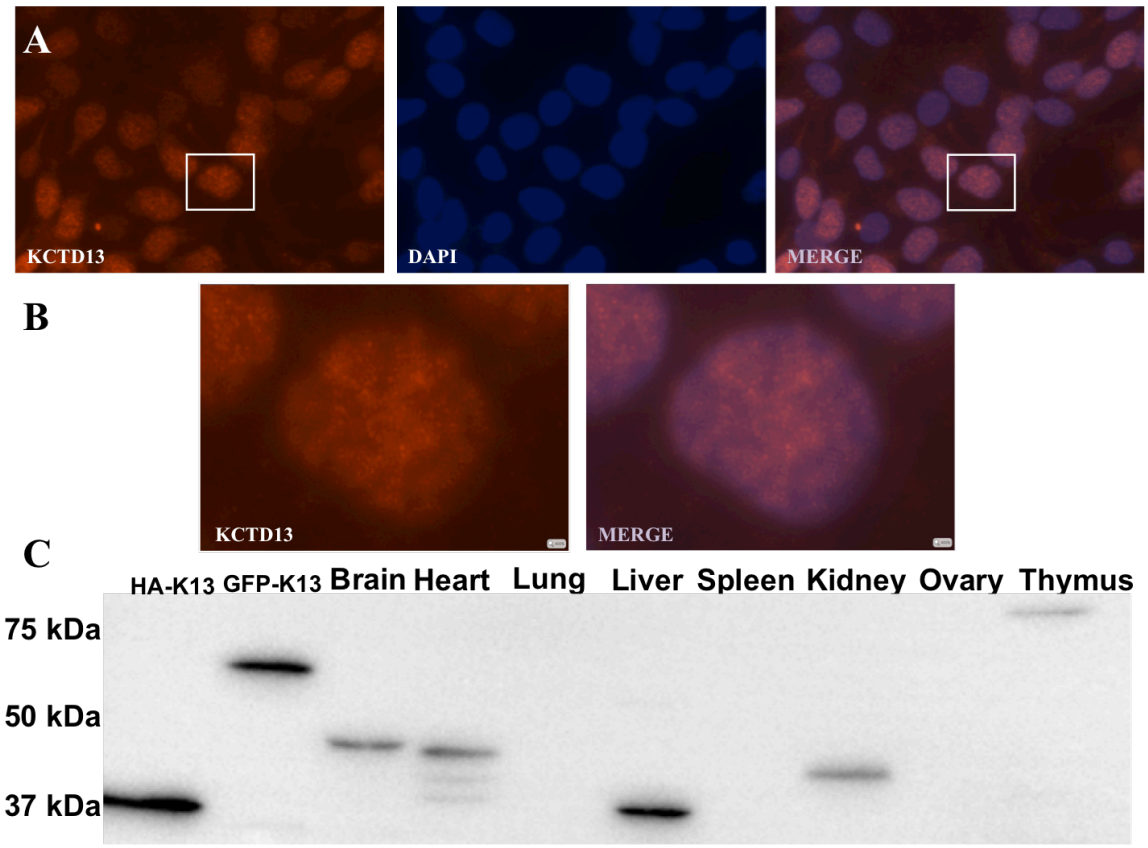

Figure 20. Endogenous KCTD13 Expression. (A) Endogenous KCTD13 expression was detected using a KCTD13 antibody from Cell Signaling. KCTD13 forms diffuse granular structures in the nucleus. **(B)** Inset of area boxed in white from Figure A shows the granular structures observed in HeLa cells stained with KCTD13 antibody. **(C)** Western Blot analysis of Wild Type 129 female mouse reveals the presence of KCTD13 in different tissues. KCTD13 is detected in tissues such as heart, lung, spleen and ovary. Consistent with its predicament in neurological disorders, KCTD13 is also expressed in the Brain.

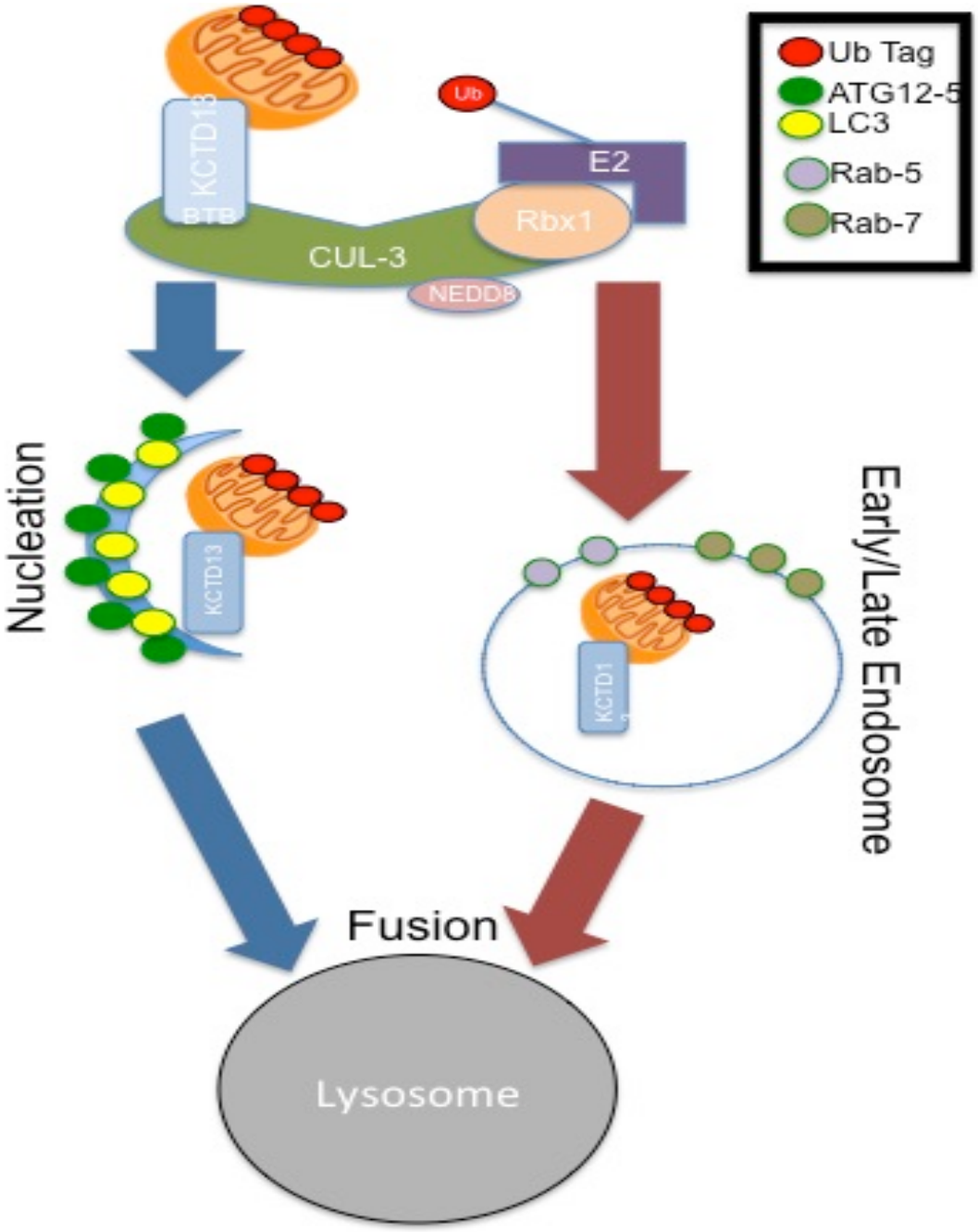

Figure 21. Model for the Potential Role of KCTD13 in the Autophagy Pathway. KCTD13 localizes with Cullin-3 and Mitochondria suggesting a role of KCTD13 in the recruitment of cargo in the autophagy pathway. The role of KCTD13 in the autophagy pathway can either lead to the delivery of mitochondria to nucleation or in endocytic pathway.

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Work Experience:

NJ Department of Health- Public Health Environmental and Agricultural Laboratories, West Trenton NJ, January 2014- May 2014
Laboratory Outreach Intern
- Plans and develops an outreach program, Laboratory Science Career day, to students aged 18-25.
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- Rotates around the different laboratories, learning basic laboratory techniques.

Behavior Therapy Associates, Somerset NJ, September 2013- January 2014
Session Assistant
- Assisted Dr. Cooperberg, Clinical Psychologist, in the running of the session
- Handed out verbal and tangible reinforcements to session participants
- Guided and interacted with session participants in group activities

Honors Tutoring, New Brunswick NJ, September 2012- January 2014
Student Tutor
- Tutored General Biology, Pre-Calculus, General Psychology, Physiological Psychology and Fundamentals of Cell Biology

Puerto Rican Action Board (PRAB), New Brunswick NJ, September 2013- December 2013
Student Intern- Head start division
- Assisted classroom teachers in the normal day-to-day activities within the classroom.
- Taught preschool students lessons such as reading, writing, art and simple math
- Encouraged and participated in small group activities with children

Douglass Developmental Disabilities Center, New Brunswick NJ, January 2013- May 2013
Fieldworker
- Ran Applied Behavioral Analysis (ABA) based, self-help programs to children with Autism
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Pizzarro Medical Clinic, Jersey City, NJ, December 2009
Volunteer
- Organized medical equipment and medications
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Research Experience:

Hardwick Laboratory, Baltimore MD, November 2014- Present
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- Characterizes a novel autophagy protein through mammalian cell transfection and immunofluorescence microscopy.
- Characterizes a mice model for seizure through PCR genotyping, mice breeding and seizure tests

Institute for the Study of Child Development, New Brunswick NJ, September 2013- January 2014
Research Assistant- Sesame Street Resilience Project Laboratory
- Encoded and Analyzed pre-test and post-test data using excel and SPSS
- Assisted in recruitment and maintenance of new research subjects
- Searched online literature sources such as PubMed, Google Scholar, Elsevier, and Ebsco for journal references.
- Assisted in office related work

Social Cognition Laboratory, New Brunswick, NJ, September 2011-May 2012

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